The effect of histone deacetylase inhibitors on gene expression in breast cancer cell lines

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ABSTRACT

Histone deacetylase inhibitors (HDIs) are a novel class of chemotherapeutics that have potent anti-proliferative and cytotoxic properties in many cancer-derived cell lines. Research has demonstrated that these compounds can activate genes such as the cell cycle inhibitor $p21^{WAF1}$, while repressing the SRC and MYC proto-oncogenes. To investigate the effects of several HDI compounds in a panel of breast cancer-derived cell lines, a reverse transcriptase qPCR (RT-qPCR) and immuno-blotting approach was used. These compounds corresponded to various classes of these therapeutic drugs, and include Trichostatin A (TSA), Apicidin, Entinostat and Mocetinostat, while the cell lines were representative of the heterogeneity of breast cancer. It was hypothesised that while these drugs demonstrate similar cellular responses such as enhanced histone acetylation, cytotoxicity and $p21^{WAF1}$ induction, they have different effects on their ability to repress genes. Using qPCR techniques, the expression of the $p21^{WAF1}$, SRC and MYC was analysed following HDI treatment in four cell lines. SRC repression were observed in all cell lines following TSA and Apicidin treatment, whereas the effects of Entinostat and Mocetinostat were more diverse; these compounds had no effect or induced expression of SRC in T47D, Hs578T and HCC-1419 cell lines while repressing expression in the BT-474 cell line. The expression of MYC was down-regulated with TSA only in T47D and BT474 cell lines, while Apicidin, Entinostat and Mocetinostat induced expression in all cell lines. However, these four inhibitors induced $p21^{WAF1}$ while exhibiting cytotoxicity and histone acetylation. In addition, it has been illustrated in the literature that RNA polymerase II-transcribed miRNA can be epigenetically modulated by chromatin-remodelling drugs. Therefore, the expression of tumour suppressor miRNA was analysed following drug treatment, and it was observed that HDIs up-regulated the expression of certain miRNAs in a cell-specific manner. miR-129-5p was induced with TSA and Entinostat in the T47D cell line, while miR-424 increased following TSA, Entinostat and Mocetinostat treatment in T47D and Hs578T cell lines. In addition, TSA induced expression of miR-9-3p in T47D, Hs578T and HCC-1419 cell lines. It was further determined that induction of these miRNA genes down-regulated the protein and/or mRNA expression of their target genes. The data presented in this thesis highlight the complex nature and the myriad effects of these inhibitors, and suggest that certain chemotherapeutics might have a clinical advantage over others in treating certain types of breast cancer.

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LIST OF ABBREVIATIONS

Ago	argonaute
ATCC	American type culture collection
AZA	5-aza-2'deoxycytidine
BR	basic region
BRCA1	breast cancer susceptibility gene 1
Cas	Crk-associated substrate
CDK	cyclin-dependent kinase
cDNA	complementary DNA
ChIP	chromatin immuno-precipitation
CIP1	CDK-interacting protein-1
CKI	cyclin-dependent kinase inhibitors
CLL	chronic lymphocytic leucaemia
CoREST	co-repressor to RE1 silencing transcription factor
co-IP	co-immuno-precipitation
DAD	deacetylase activating domain
DNMT	DNA methyltransferase
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EtOH	ethanol
ERα	oestrogen receptor alpha
ERβ	oestrogen receptor beta

ERE	oestrogen response element
ERBB2	erythroblastosis oncogene B2
FBS	foetal bovine serum
G1	growth phase 1
G2	growth phase 2
H3Ac	histone H3 acetylation
H3K4me^3	histone H3 lysine 4 tri-methylation
H3K9Ac	histone H3 lysine 9 acetylation
H3K9me^3	histone H3 lysine 9 tri-methylation
H3K36	histone H3 lysine 36
H3S10	histone H3 serine 10
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDI	histone deacetylase inhibitor
Hda1	histone deacetylase I
HER2/*neu*	human epidermal growth factor receptor 2/*neu*
HIF-1α	hypoxia inducible factor 1α
Hsp90	heat shock protein 90
HNF-1	hepatocyte nuclear factor 1
IP4	inositol tetraphosphate
KAT	lysine acetyltransferases
KDAC	lysine deacetylases
M phase	mitotic phase
MAPK	mitogen -activated protein kinase
MBI	Myc homology box I

MBII	Myc homology box II
MEF2	myocyte enhancer factor 2
miRNA	microRNA
MMP-2	metalloproteinase-2
MMTV	mouse mammary tumour virus
MRE	miRNA response elements
mRNA	messenger RNA
NaB	sodium butyrate
NAD^+	nicotine adenine dinucleotide
NCoR/SMRT	nuclear receptor co-repressor/silencing mediator of retinoic and thyroid receptors
NELF	negative elongation factor
NuRD	nucleosome remodelling histone deacetylase/chromodomain 3
NF-κB	nuclear factor κB
PBS	phosphate buffered saline
PBST	phosphate buffered saline and TWEEN-20
PDGFR	platelet-derived growth factor receptor
PgR	progesterone receptor
PP2A	protein phosphatase 2A
PP4	protein phosphatase 4
Pri-miRNA	primary miRNA
Pre-miRNA	precursor miRNA
RT-qPCR	reverse transcriptase quantitative PCR
RARE	retinoic acid-responsiveness elements
pRb1	retinoblastoma protein

RECK	reversion-inducing-cysteine-rich protein with kazal motifs
RISC	RNA-induced silencing complex
RNA pol II	RNA polymerase II
ROS	reactive oxygen species
Rpd3	reduced potassium dependency 3
RTK	receptor tyrosine kinases
RUNX	runt domain transcription factor
S phase	DNA synthesis phase
SAHA	suberoylanilide hydroxamic acid
SFK	Src family kinases
SH2	Src homology domain 2
SH3	Src homology domain 3
SH4	Src homology domain 4
Sir2	silent information regulator 2
Sin3A/B	switch independent 3A/B
STAT	signal transducer and activator of transcription
stRNA	short temporal RNA
TAD	amino-terminal transactivation domain
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFα	transforming growth factor α
TRAIL	TNF-related apoptosis-inducing ligand
TSA	trichostatin A
TSS	transcription start site
UTR	untranslated region
WAF1	wild-type associated factor-1

1. REVIEW OF THE LITERATURE

1.1 Introduction

Malignancies can develop through dysfunctional cellular processes that result from inactivation of tumour suppressor genes and/or activation of oncogenes (Stearns *et al*., 2007; Podo *et al*., 2010). These can arise through many processes, including epigenetic modifications such as promoter CpG methylation by DNA methyltransferases and imbalances in histone acetylation by histone acetyltransferases and histone deacetylases (HDACs). The dysregulation of epigenetic mechanisms represents a level of gene control that can be exploited by chromatin-remodelling chemotherapeutics. Within Canada, it was estimated that ninety thousand women would develop cancer in 2014, with one in every nine women having a risk to develop breast cancer in their lifetimes (Canadian Cancer Statistics, 2014). Histone deacetylase inhibitors (HDIs) are a novel class of chemotherapeutics that preferentially affect cancerous cells, leading to apoptosis, cell cycle arrest and differentiation (Mehnert *et al*., 2007; Ma *et al*., 2009; Wanczyk *et al*., 2011; Nebbioso *et al*., 2010). It is traditionally thought that HDI treatment facilitates histone acetylation and transcriptional activation through inhibiting HDAC enzymes. However, only a small fraction of genes are up-regulated in this manner; in fact, many genes are also repressed upon drug treatment (Van Lint *et al*., 1996; Gray *et al*., 2004; Marks *et al*., 2004; Peart *et al*., 2005; Ropero and Esteller, 2007; LeBonte *et al*., 2009; Halsall *et al*., 2012; Khan *et al*., 2012). The SRC and MYC proto-oncogenes are over-expressed in many cancers, including those of the breast (Escot *et al*., 1986; Wong *et al*., 1986; Dubik *et al*., 1987; Blanchard *et al*., 1988; Cartwright *et al*., 1989, 1990; Brunton *et al*., 1997; Iravani *et al*., 1998; Dehm and Bonham, 2004; Alvarez *et al*., 2006; Chen and Olopade, 2008; Wheeler *et al*., 2009; Horiuchi *et al*., 2012), and are repressed following HDI treatment (Kostyniuk *et al*., 2002; Dehm and Bonham, 2004; Hirsch *et al*., 2006; Bonham and Beaton-Brown, unpublished data). While these mechanisms are currently unknown, it likely involves the adoption of a promoter proximal paused state which is associated with the loss of histone H3 phosphorylation, recruitment of the negative elongation factor NELF and further de-phosphorylation of the CTD region of RNA polymerase II (RNA pol II) and loss of the complex from the gene body (Bonham and Beaton-Brown, unpublished data). Furthermore, cellular processes affected by HDIs are often myriad and cell-specific; for example,

certain chemical classes of HDIs affect cell cycle arrest and apoptosis in a differential manner. In addition, only a small percentage of genes are similarly regulated by the same HDI (Halsall *et al*., 2012; Chatterjee *et al*., 2013). It has also been shown that microRNA (miRNA) transcribed by RNA pol II are induced or repressed following HDI administration (Lu *et al.,* 2005; Scott *et al.,* 2006). These non-coding miRNAs control numerous cellular processes important in the pathogenesis of many malignancies, including cancer (Iorio *et al.,* 2005; Lu *et al.,* 2005; Scott *et al.,* 2006; Lowery *et al.,* 2009). The experiments presented in this thesis investigated differential HDI-mediated effects on histone acetylation, cell viability and gene expression in four breast cancer cell lines representative of the heterogenous nature of breast cancer. Furthermore, the effects of these compounds upon miRNA expression and subsequently miRNA-mediated gene and protein regulation were explored. The data presented demonstrate divergences between the effects of these drugs in diverse breast cancer subtypes. This literature review briefly summarises vital aspects of the pathological and molecular subtypes of breast cancer and associated therapeutics. The current knowledge of HDACs and HDIs are then reviewed, followed by a detailed background in the context of cancer and transcriptional control on $p21^{WAF1}$ and the proto-oncogenes SRC and MYC. Finally, miRNA and their associated cellular regulatory processes are discussed.

1.2 Breast Cancer

Malignant tumours arise through the accumulation of defects in cellular regulatory processes, including metabolic alteration, evasion of the cell cycle control checkpoints, faulty DNA repair mechanisms, apoptotic defects and invasive properties leading to metastasis. These dysfunctional processes can result from the silencing and inactivation of tumour suppressor genes and/or the activation of oncogenes (Lengauer *et al*., 1998; Hanahan and Weinberg, 2000; Volgstein and Kinzler, 2004). Multiple levels of inactivation and activation can arise through translocations, mutations, amplifications, deletions and/or epigenetic alterations, such as promoter methylation and/or chromatin remodelling via altered histone acetylation. Epigenetic mechanisms represent an alternative method of gene control, which does not alter the nucleotide sequence. The most commonly studied of these is the methylation of CpG dinucleotides in promoter regions, enzymatically carried out by DNA methyltransferases (DNMTs) in dividing

cells. Another well-known epigenetic modulation is post-translational histone modifications, some of which are mediated by histone deacetylase (HDAC) enzymes (Lengauer *et al*., 1998; Hanahan and Weinberg, 2000; Volgstein and Kinzler, 2004; Stearns *et al*., 2007; Elias, 2010; Podo *et al*., 2010; Timp and Feinberg, 2013), which are the focus in a forthcoming section.

Breast carcinoma is considered a group of clinically and pathologically heterogeneous malignancies, and attempts to cluster these diseases into homogenous entities have only been partially successful (Elias, 2010). Annually, over one and a half million women worldwide are diagnosed with breast tumours, and it has become the most common cancer in women (Bouchalova *et al*., 2009; Tate *et al*., 2012; Ferlay *et al*., 2015). In a statistical report released by the Canadian Cancer Society, it was estimated that ninety thousand women developed cancer in 2014, with twenty-six percent of these cases representing new diagnoses of breast cancer. Moreover, one in every nine women has the potential to develop the disease in their lifetime (Canadian Cancer Statistics, 2014).

1.2.1 Pathological and Molecular Classes of Breast Cancer

There are two common histological categories of breast carcinoma, ductal and lobular (Bouchalova *et al*., 2009; Malhotra *et al*., 2010). Lobular carcinomas develop from the lobules glands in the breast, whereas ductal carcinomas originate from the lactiferous ducts and are the most frequently diagnosed histological pattern. Both ductal and lobular carcinomas can remain *in situ* or become invasive and migrate from their sites of origin. In fact, invasive ductal carcinoma encompasses various subtypes, including medullary carcinoma, mucinous carcinoma and papillary carcinoma (Malhotra *et al*., 2010).

Breast cancers have historically been classified based on histological and morphological features, as well as by the 'TNM' system consisting of tumour size, lymph node status and metastatic occurrence. Based on this classification, breast tumours were categorised into eighteen histological subtypes, which failed to form homogeneous clusters (Elston and Ellis, 1993; Tavassoli and Devilee, 2003; Weigelt *et al*., 2008; de Ruijter *et al*., 2010; Malhotra *et al*., 2010). DNA microarray analysis has provided an additional method for breast cancer classification, based upon gene expression profiling of the transcriptome. This has further illustrated its heterogeneous nature (Sorlie *et al*., 2001); gene expression profiling does not form

homogeneous clusters similar with the historical histo-morphological clustering. Therefore, breast cancer is considered a heterogeneous disease with considerable phenotypic and genetic diversity (Perou *et al*., 2000; Sorlie *et al*., 2001; de Ruijter *et al*., 2010), several of which are discussed shortly.

Following diagnoses, patient prognosis is determined based on several factors, such as tumour size and grade, lymph node metastases, vascular tumour invasion, amplification of the HER2/*neu* oncogene, and the presence or absence of oestrogen receptor-alpha (ERα) and progesterone receptor (PgR) (Table 1.1). The most striking division of breast cancer is between the presence or absence of these hormone receptors; those breast cancers which are positive for ERα and PgR expression can be further divided into 'luminal A' and 'luminal B' based on the amplification of the erythroblastosis oncogene B2 (ERBB2) gene and its protein product, the human epidermal growth factor receptor 2 (HER2/*neu*). Luminal A cancers have normal HER2/*neu* expression, whereas luminal B have enriched HER2/*neu* expression (Sorlie *et al*., 2001, 2003; de Ruijter *et al*., 2011; Renoir, 2012). In contrast, the basal subtype is most often triple-negative breast tumours and therefore lacks ERα and PgR expression, and HER2/*neu* is not amplified and/or over-expressed. The last major subtype based on gene expression profiling is the HER2-amplified category, which is frequently negative for ERα and PgR expression but possesses amplified HER2/*neu* as the nomenclature suggests (Sorlie *et al*., 2001, 2003; Bertucci, 2008; de Ruijter *et al*., 2010; Renoir, 2012). In addition, certain oncogenic mutations have been shown to cluster with either ERα-positive or ERα-negative cancers, such as mutations in the PTEN and Rb1 tumour suppressors which associate with triple-negative breast cancers (Hu *et al*., 2009; de Ruijter *et al*., 2010). While these aforementioned divisions are beneficial for prognostic value, they also assist in determining clinical therapeutic options and predict patient response (Bouchalova *et al*., 2009; Renoir, 2012).

1.2.2 Breast Cancer Therapy

The identification of the ERα and HER2/*neu* molecular markers has facilitated the movement toward personalised therapy targeted against ERα expression and HER2/*neu* amplification (Bouchalova *et al*., 2009; Podo *et al*., 2010; Renoir, 2012). Personalised therapy utilises molecular signatures, biological markers and clinicopathological features of the tumours

Table 1.1. The common molecular subtypes of breast cancer. Luminal A and B express the hormone receptors ERα and PgR, whereas expression is absent in basal and HER2-amplifed. The HER2/*neu* protein and/or gene is amplified only in Luminal B and the HER2-amplified subtypes.

	ERα and PgR	**HER2/*neu***
Luminal A	present	normal
Luminal B	present	amplified
Basal	absent	normal
HER2-Amplified	absent	amplified

to treat patients. This method of treatment interferes with specific molecules highly expressed in the cancerous tissue and is relatively ineffective in normal cells (de Ruijter *et al*., 2010; Podo *et al*., 2010). Numerous biomolecules such as metabolites, proteins and enzymes are considered candidate biomarkers in breast cancer pathogenesis. The first personalised anti-hormonal therapies for breast cancer were targeted against ERα and/or PgR, whereas the identification of the HER2/*neu* gene was the first cytogenic marker (Bouchalova *et al*., 2009; Podo *et al*., 2010). The presence of ERα and PgR, and HER2/*neu* amplification are the subdivisions of breast cancer clinicians frequently employ, resulting in three main subtypes: ERα-positive, HER2-amplified and triple-negative. These subtypes are further stratified based on their therapeutic potential and prognoses (Elias, 2010) and are briefly discussed below.

1.2.2.1 ERα-Positive Breast Cancers

In normal development, oestrogens control the physiological growth and differentiation of normal mammary tissue through the actions of the ERα and oestrogen receptor beta (ERβ). These are members of the nuclear receptor superfamily of transcription factors and possess a carboxy terminal ligand-binding domain necessary for activation and dimerisation. In response to the presence of oestrogens, dimerisation results in a conformational change, allowing interaction with numerous transcription regulators. These multi-protein complexes bind to ER-responsive elements and alter chromatin structure, ultimately leading to altered gene expression (Jenuwein and Allis, 2001; Sommer and Fuqua, 2001; Margueron *et al*., 2004; Pathiraja *et al*., 2010; Renoir, 2012).

Through the action of ERα and ERβ, oestrogens act as mitogens and control numerous cellular functions critical in hormone-responsive carcinogenesis (Henderson *et al*., 1982; Conzen 2008; Cicatiello *et al*., 2010; Pathiraja *et al*., 2010). While the function of ERβ in breast cancer progression is relatively unknown, the role of ERα has been extensively studied (Pathiraja *et al*., 2010; Renoir, 2012). Oestrogens alter gene expression in ERα-positive luminal A and luminal B cell lines (Cicatiello *et al*., 2010), and inhibiting these pathways is therapeutically beneficial (Cicatiello *et al*., 2010; de Ruijter *et al*., 2010). In fact, signalling from ERα is one of the significant determinants of tumorigenesis in ERα-positive breast cancers, which accounts for approximately sixty-five to seventy percent of breast cancer diagnoses (Thomas *et al*., 2011).

With the advent of individualised therapy, these oestrogen-dependent, ERα-positive cancers can be treated with anti-hormonals (Swain *et al*., 2005; Goss *et al*., 2008; Thomas and Munster, 2009; de Ruijter *et al*., 2010; Podo *et al*., 2010; Sabnis *et al*., 2011; Thomas *et al*., 2011). The preferred treatment for hormone-sensitive cancers is targeting ERα through anti-oestrogen molecules, although less than fifty percent of ERα-positive cases continually respond to endocrine therapy; *de novo* and acquired resistance occur in a quarter of ERα-positive tumours. This resistance is thought to result from transcriptional inactivation of ERα through mechanisms such as promoter methylation (Katzenellenbogen, 1991; Ottaviano *et al*., 1994; Thomas and Munster, 2009; Zilli *et al*., 2009; Pathiraga *et al*., 2010; Osborne *et al*., 2011; Retnoir, 2012). Tamoxifen, through its conversion to 4-hydroxytamoxifen, binds to ERα and competes with oestrogen binding, thus preventing ERα-dependent signalling. This alters the expression of oestrogen-responsive genes and generally inhibits oestrogen-dependent cellular growth (Thomas and Munster, 2009; Thomas *et al*., 2011). In addition, aromatase inhibitors can be exploited to reduce the synthesis of oestrogen from testosterone and androenedione through the activity of the enzyme aromatase (Pathiraja *et al*., 2010; Retnoir, 2012).

Several reports have indicated that ERα expression can be modulated through chromatin remodelling drugs such as DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) (Yang *et al*., 2000; Keen *et al*., 2003; Yan *et al*., 2003; Stearns *et al*., 2007; Bicaku *et al*., 2008; Stark *et al*., 2013). While HDACs in particular are reviewed in an upcoming section, it is interesting to note that HDAC inhibition may augment the cytotoxicity of anti-oestrogen treatment (Fan *et al*., 2008; Bicaku *et al*., 2008; Thomas *et al*., 2011). Furthermore, *in vitro* cell line studies have illustrated HDAC inhibitors (HDIs) may in fact increase ERα mRNA expression in ERα-negative cells, an effect opposite to that observed within ERα-positive cells (Yang *et al*., 2000; Keen *et al*., 2003; Bicaku *et al*., 2008; Fan *et al*., 2008; Thomas *et al*., 2011). This inducing effect was also observed for PgR mRNA in ERα-negative cells, although functional ERα rather than HDI-dependent epigenetic mechanisms may be responsible (Keen *et al*., 2003; Fleury *et al*., 2008). The alteration of histone acetylation is not the sole determinant of ERα expression however, and DNMT inhibitors (alone or in conjunction with an HDI) have also restored ERα levels and re-sensitised ERα-negative cell lines to endocrine therapy (Yan *et al*., 2003). In particular, synergistic treatments of Vorinostat, Trichostatin A (TSA) or Scriptaid with the DNMT inhibitor 5-aza-2'deoxycytidine (AZA) increased ERα mRNA expression in ERα-

negative cell lines (Yang *et al*., 2000; Keen *et al*., 2003; Sharma *et al*., 2004; Jang *et al*., 2006; Stark *et al*., 2013).

Additionally, apoptotic processes are induced when breast cancer cell lines are co-treated with an HDI and tamoxifen, and thus HDAC inhibition has been considered a viable treatment option for ERα-positive cancers (Bicaku *et al*., 2008; Fan *et al*., 2008; Thomas *et al*., 2011). Furthermore, tamoxifen and Vorinostat have been used in combination to treat ERα-positive metastatic cancers and restored hormone sensitivity (Munster *et al*., 2009). In fact, many clinical and pre-clinical studies involving DNMTs and/or HDIs in conjunction with endocrine therapies are currently in progress (Bicaku *et al*., 2008; Fan *et al*., 2008; Chavan and Somani, 2010; Pathiraga *et al*., 2010; Ferguson *et al*., 2011; Martinet and Bertrand, 2011; Thomas *et al*., 2011). HDI-mediated effects upon ERα and PgR expression, as well as general non-transcriptional effects, are discussed in greater detail in a forthcoming subsection.

1.2.2.2 HER2/*neu* Amplified Breast Cancers

The receptor tyrosine kinase HER2/*neu* is amplified in approximately twenty-five to thirty percent of breast carcinomas and is generally indicative of poorer clinical outcomes, lower survival rates, increased metastasis and advanced tumour stage with aggressive phenotypes (Garcia *et al*., 1989; Chen *et al*., 2010; Tagliabue *et al*., 2010; Kodack *et al*., 2012). A high incidence of brain metastases is associated with HER2-amplified cancers, likely due to the inability of therapeutics to cross the blood-brain barrier (Kodack *et al*., 2012).

There are three homologous HER receptor tyrosine kinases (RTKs), HER1 (also known as the epidermal growth factor receptor [EGFR]) and HER3 and HER4. These RTKs hetero- or homo-dimerise and autophosphorylate conserved tyrosine moieties to recruit downstream signalling proteins. This enhances cellular proliferation through the induction of growth-promoting genes. HER2/*neu* is ligand-independent and hetero-dimerises with other HER family members in the absence of extracellular ligand, thus making it a potent oncogenic kinase (Sorlie *et al*., 2001; Hondermarck, 2003; Citri and Yarden, 2006; Chen *et al*., 2010). In fact, HER2/*neu* up-regulation is sufficient to induce tumorigenicity in a normal mammary epithelial cell line (Kim *et al*., 2009).

While personalised therapy has lead to the treatment of ERα-positive cancers with anti-oestrogens and aromatase inhibitors, HER2-amplified cancers can be targeted with trastuzumab, an antibody which targets the HER2/*neu* receptor (Swain *et al*., 2005; Goss *et al*., 2008; de Ruijter *et al*., 2010; Sabnis *et al*., 2011). Otherwise known in the market as 'Herceptin', trastuzumab is a humanised monoclonal antibody that blocks HER2/*neu* activity by targeting the extracellular domain IV (Podo *et al*., 2010). While trastuzumab has decreased breast cancer-related mortality, a significant fraction of patients develop resistance or refractory disease, while those with brain metastatic disease are incurable. A secondary avenue of therapy, the humanised monoclonal antibody pertuzumab (Perjeta), targets the dimerisation domain of HER2/*neu*. This prevents dimerisation and has resulted in favourable patient prognosis in clinical trials. In addition, trastuzumab and pertuzumab have exhibited promising results when used in conjunction (Piccart-Gebhart *et al*., 2005; Perez *et al*., 2011; Slamon *et al*., 2011; Lamond and Younis, 2014; O'Sullivan and O'Connolly, 2014).

Interestingly, synergistic treatment of HER2-positive cell lines with the sodium butyrate (NaB) and trastuzumab enhanced the anti-proliferative effects of the drugs. This effect was insignificant in HER2-negative cell lines (Chen *et al*., 2007). In addition, Vorinostat attenuated HER2/*neu* expression in HER2-positive breast cancer cell lines, and dual-treatment with trastuzumab further decreased cellular growth (Bali *et al*., 2005).

1.2.2.3 Triple-Negative Breast Cancers

Similar to the HER2-amplified breast cancers, triple-negative account for fifteen to twenty percent of breast cancer diagnoses, and are frequently classified as high-grade invasive ductal carcinomas (Bouchalov *et al*., 2009; Elias, 2010). Triple-negative cancers are often associated with an aggressive tumour phenotype and poor prognosis with higher distant recurrence rates and reduced overall survival (de Ruijter *et al*., 2010; Podo *et al*., 2010). Patients presenting with triple-negative cancers are generally younger in age than those diagnosed with ERα-positive cancers, and generally have higher mortality rates. In addition, there is a higher incidence of triple-negative cancers among those of African-American descent (Dent *et al*., 2007; Rakha *et al*., 2007; Bouchalov *et al*., 2009; Elias, 2010; Podo *et al*., 2010).

While these cancers are often synonymous with the basal subtype, molecular analysis has

shown that these are two separate entities. In general, the basal-like, triple-negative cancers present with more chromosomal losses and/or gains than the luminal subtypes (Podo *et al*., 2010). Cytogenetics have also revealed genomic fragile sites common in triple-negative breast cancers, such as amplification of the 8q24 and 17q12 loci, corresponding to the MYC and HER2/*neu* genes (Struski *et al*., 2002; Bouchalova *et al*., 2009). MYC is a transcription factor thought to control approximately fifteen percent of human genes and its amplification is associated with poorer prognosis (Escot *et al*., 1986; Dubik *et al*., 1987; Garcia *et al*., 1989; Nass and Dickson, 1997; Levens, 2008; Chen and Olopade, 2008; Horiuchi *et al*., 2012). In addition, ERα-negative breast cancers have been shown to have higher proliferative rates than ERα-positive cancers, a characteristic which is often associated with MYC amplification (Wirapati *et al*., 2008). MYC and its role in carcinogenesis are discussed in greater detail in an upcoming section. In addition, expression of EGFR and mutations in PTEN were more common in triple-negative breast cancers than other subtypes (Elias, 2010). The association of mutations in the BRCA1 and BRCA2 genes in triple-negative cancers are of particular interest; these familial breast cancer predisposing genes are involved with DNA repair and can be exploited in breast cancer treatment (Podo *et al*., 2010).

Due to the lack of HER2/*neu* over-expression and absence of ERα and PgR, the antibody trastuzumab and endocrine therapies are unsuccessful in treating these breast cancers. In addition, they currently lack targeted clinical therapies and are partially resistant to treatment. However, one avenue of therapy for these breast cancers are cytotoxic chemotherapies that induce DNA damage (Elias, 2010; Podo *et al*., 2010). The mutation of BRCA1 previously mentioned to be correlated with the triple-negative subtype suggests that cisplatin and/or carboplatin are promising treatments for these breast cancers (Silver *et al*., 2010; Elias, 2010; Podo *et al*., 2010; Valero, 2014; von Minckwitz, 2014). Cytotoxic therapies such as paclitaxel and cyclophosphamide have also shown favourable results against triple-negative cancers (Podo *et al*., 2010). In addition, agents inhibiting the activity of tyrosine kinases such as pp60 c-Src have promising pre-clinical results in cancer treatments (Finn *et al*., 2007; Elias, 2010).

In addition, the HDI panobinostat has been shown to be cytotoxic to triple-negative cell lines and able to reduce tumour growth *in vivo*. This was associated with a partial mesenchymal-to-epithelial transition in morphology, indicative of diminished invasive properties (Tate *et al*., 2012). However, while HDIs induce apoptosis and promote autophagy, cancer cells with

defective apoptotic systems can survive treatment (Shao *et al*., 2004). Combining an autophagy inhibitor with panobinostat has demonstrated reduced tumour burden and increased survival in mouse xenograft models of triple-negative breast cancer (Rao *et al*., 2012). Dual therapy of Vorinostat with a kinase inhibitor or receptor antagonist further potentiated anti-tumour effects of the chemotherapeutics, indicating novel therapeutic strategies involving HDAC inhibitors (Fiskus *et al*., 2012; Stark *et al*., 2013). Furthermore, while triple-negative cancers remain difficult to treat due to a lack of targeted therapies, HDI treatment of ERα-negative cell lines and derived xenografts re-sensitise these tumours to hormone treatment. This is partially through activating ERα expression, where anti-oestrogens and aromatase inhibitors are then rendered effective (Jang *et al*., 2004; Margueron *et al*., 2004; Li *et al*., 2010; Munster *et al*., 2011; Gryder *et al*., 2013; Stark *et al*., 2013).

1.2.3 Summary

Although breast carcinoma related deaths have been decreasing since the 1980s, with the morality rate falling approximately 40%, it remains the second leading cause of cancer-related deaths among women in Canada (Canadian Cancer Statistics, 2014). In the clinic, patient tumours are stratified according to their histolo-morphological features and gene expression patterns, and these subtypes determine clinical therapy and are able to predict patient outcome (Bouchalova *et al*., 2009; Podo *et al*., 2010; Renior 2012). ERα-positive disease accounts for the majority of diagnoses, while HER2-amplified and triple-negative account for the remaining fifteen to twenty percent (Tagliabue *et al*., 2010; Thomas *et al*., 2011; Kodack *et al*., 2012). While ERα-positive and HER2-amplified tumours can be treated with anti-hormonals and receptor-targeted antibodies, personalised therapies remain ineffective against triple-negative cancers (de Ruijter *et al*., 2010; Podo *et al*., 2010; Sabnis *et al*., 2011; Thomas *et al*., 2011). As previously mentioned, one mechanism by which ERα transcription is silenced in ERα-negative tumours is via histone modification and/or promoter methylation. Histone acetylation is generally associated with active transcription, and manipulating the acetylation status is a viable therapeutic option for many cancers, as briefly highlighted (Yang *et al*., 2000; Keen *et al*., 2003; Jang *et al*., 2004; Sharma *et al*., 2006; Tsai and Baylin, 2011; Stark *et al*., 2013). This dynamic process is controlled by a set of reciprocally-acting enzymes known as HDACs and histone

acetyltransferases (HATs), proteins which are frequently deregulated in numerous carcinomas, including those of the breast (Allfrey, 1964; Roth *et al*., 2001; Khorasanizadeh, 2004; Sjoblom *et al*., 2006; Ma *et al*., 2009; Bannister and Kouzarides, 2011; Wanczyk *et al*., 2011; Barneda-Zahonero and Parra, 2012; Timp and Feinberg, 2013). While examples of HDI administration in breast cancer have been described earlier, a more detailed analysis of the HDAC enzymes and the consequences of their inhibition follows.

1.3 Histone Deacetylases

In eukaryotic organisms, genomic DNA is packaged into highly structured chromatin fibres within the nucleus. The main component of chromatin is the nucleosome, DNA segments of 147 base-pairs wrapped around the core histone octamer, which consists of two copies of histones H2A, H2B, H3 and H4 (Wolffe, 1994; Khorasanizadeh, 2004; Barneda-Zahonero and Parra, 2012). The amino-terminal histone tails that extend from the nucleosomes are enriched with lysine moieties, and the positive charge facilitates histone-histone and/or histone-DNA ionic interactions (Hebbes *et al*., 1988; Ridsdale *et al*., 1990; Kelly and Cowley, 2013). Epigenetic, post-translational histone modifications have the ability to modify these chromatin structures, subsequently controlling transcriptional activation or repression (Khorasanizadeh, 2004; Ropero and Esteller, 2007; Barneda-Zahonero and Parra, 2012). These include the phosphorylation of serine or tyrosine residues, the methylation of arginines, and the ubiquitination, methylation, sumoylation or acetylation of lysines (Khorasanizadeh, 2004; Mehnert and Kelly, 2007; Bannister and Kouzarides, 2011; Barneda-Zahonero and Parra, 2012).

Histone acetylation and deacetylation are reversible enzymatic reactions controlled by the reciprocally-acting histone acetyltransferases (HATs) and HDACs. While HATs catalyse the addition of an acetyl group from acetyl-CoA to the epsilon-amino group of lysines, HDACs act to remove the acetyl group as an acetate molecule (Allfrey, 1964; Roth *et al*., 2001; Khorasanizadeh, 2004; Santos-Rosa *et al*., 2005; Bannister and Kouzarides, 2011; Barneda-Zahonero and Parra, 2012). This acetylation neutralises the positive charge of the lysine residue, minimising ionic interactions and resulting in a relaxed or ‘open’ chromatin conformation (called ‘euchromatin’) that facilitates transcription factor binding (Felsenfeld, 1992; Wolffe, 1994; Ueda *et al*., 2006; Ropero and Esteller, 2007; Haberland *et al*., 2009; Wang *et al*., 2010; Barneda-

Zahonero and Parra, 2012). Histone acetylation also decreases association with the linker histone H1 (Kelly and Cowley, 2013) and forms a binding site for bromodomain-containing proteins (Halsall *et al*., 2012; Kelly and Cowley, 2013); therefore, acetylation is generally associated with transcriptionally active genomic regions. A condensed or 'closed' chromatin conformation, referred to as 'heterochromatin', is associated with higher nucleosome density. This prevents the formation of pre-initiation complexes and access to transcriptional machinery and is thus associated with inactive genes (Felsenfeld, 1992; Wolffe, 1994; Ropero and Esteller, 2007; Haberland *et al*., 2009; Barneda-Zahonero and Parra, 2012; Halsall *et al*., 2012; Timp and Feinberg, 2013). The proteins which modify chromatin structure are generally known as 'writers' of histone acetylation, whereas proteins which recognise these chromatin markers are referred to as 'readers'. For instance, the acetylation of histone H3 lysine 9 (H3K9Ac) and the tri-methylation of histone H3 lysine 3 ($H3K4me^3$) 'written' by HAT enzymes are associated with active transcription. These chromatin marks are then 'read' by the bromodomain-containing proteins and/or transcription factors to result in transcriptional activation (Ropero and Esteller, 2007; Tsai and Baylin, 2011; Marmorstein and Zhou, 2014).

In addition to their effects on histone deacetylation, HDACs also remove acetyl groups from lysine residues on an ever-increasing amount of non-histone cytosolic and nuclear proteins. Currently, approximately 1700 of these proteins have been identified, and a single protein could have two or more lysines potentially targeted for acetylation (Kim *et al*., 2006; Peng and Seto, 2011; Barneda-Zahonero and Parra, 2012; Kelly and Cowley, 2013). Due to this, HATs and HDACs are becoming more known as lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) in the literature (Roth *et al*., 2001; Santos-Rosa *et al*., 2005; Allis *et al*., 2007), but will be referred to as 'HATs' and 'HDACs' in the forthcoming sections. Furthermore, histone acetylation is also associated with chromatin assembly, DNA damage response and recombination (Vidanes *et al*., 2005; Polo *et al*., 2005; Ropero and Esteller, 2007). These non-histone targets and physiological effects of HDACs are discussed following a brief introduction into the mechanistic action and biology of HDAC enzymes.

1.3.1 Mechanisms of HDAC Action and Structural Classes of HDACs

Currently, there are eighteen human HDAC enzymes divided into four categories based

on their sequence homology to yeast HDACs, as well as their catalytic mechanisms (Figure 1.1). Classes I, II and IV require a zinc divalent cation (Zn^{2+}) for their deacetylase activity, while the third class requires nicotine adenine dinucleotide (NAD^{+}) for catalysis (Thiagalingam *et al*., 2003; Ropero and Esteller, 2007; Xu *et al*., 2007; Schrump, 2009; Wanczyk *et al*., 2011). The Zn^{2+}-dependent HDACs have an active site with adjacent histidine and aspartate residues, a tyrosine (or histidine) residue and a Zn^{2+} ion (Bressi *et al*., 2007; Delcuve *et al*., 2013). HDACs exert their effects through nucleophilic attack of a zinc-activated H_2O molecule on the carbonyl carbon of the substrate acetyl group, resulting in an oxyanion intermediate. The negative charge of the intermediate is stabilised through interactions of the Zn^{2+} ion and the tyrosine at the active site. The resulting acetate molecule and de-acetylated lysine protein substrate are produced by proton transfer (Finnin *et al*., 1999; Vannini *et al*., 2007; Shuetz *et al*., 2008).

1.3.2 Structural Classes of HDACs

The class I HDACs (1, 2, 3 and 8) share sequence homology to the yeast transcriptional regulator Rpd3. They are ubiquitously expressed in all tissues and predominantly localised to the nucleus, with the exception of the nuclear and cytosolic HDAC3 (Yang *et al*., 2002; Mehnert and Kelly, 2007; Khan and La Thangue, 2012). Class I HDACs possess a nuclear localisation signal and are low molecular weight molecules, between 22 kDa and 55 kDa (Mehnert and Kelly, 2007; Khan and La Thangue, 2012). They have the highest catalytic rates and share similar protein structures, containing a conserved deacetylase domain, an amino-terminal extension and a carboxy-terminal extension (Yang *et al*., 2008; Nebbioso *et al*., 2010; Segre *et al*., 2011; Wanczyk *et al*., 2011; Barneda-Zahonero and Parra, 2012; Kelly and Cowley, 2013); in particular, HDACs 1 and 2 share 83% sequence identity between them, while HDAC3 only shares 53% identity to HDACs 1 and 2 (Kelly and Cowley, 2013), containing a variable carboxy terminal region non-homologous to other HDACs. (Karagianni and Wong, 2007). Interestingly, this region is also required for its deacetylase activity (Yang *et al*., 2002).

The class II HDACs are based on their domain organisation and sequence similarity to the yeast protein Hda1, and are further divided according to their structures and compartmentalisation into subgroup IIa (HDACs 4, 5, 7 and 9) and subgroup IIb (HDACs 6 and 10) (Grozinger *et al*., 1999; Bieliauskas and Pflum, 2008; Schrump, 2009; Nebbioso *et al*., 2010; Bannister and Kouzarides, 2011; Wanczyk *et al*., 2011; Barneda-Zahonero and Parra, 2012).

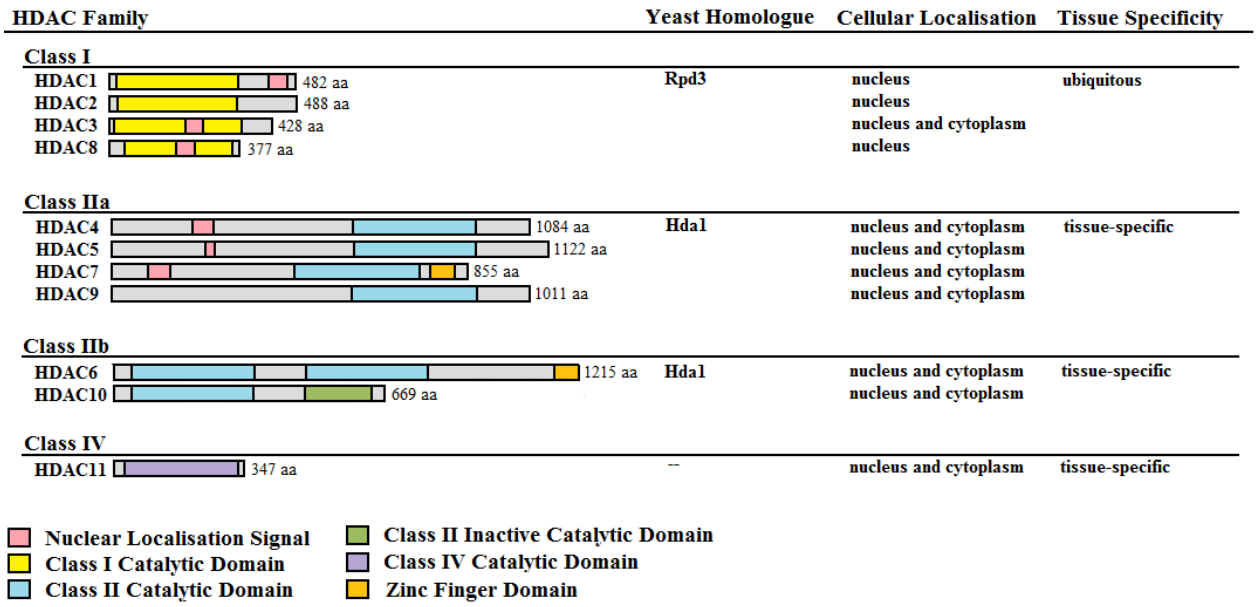

Figure 1.1. Schematic representations of the structures and cellular localisations of the human histone deacetylase enzymes. The ubiquitous class I HDACs (1, 2, 3 and 8) share structural homology to the yeast transcriptional regulator Rpd3 and are located in the cytoplasm and/or nucleus. The tissue-specific class II HDACs are homologous to the yeast protein Hda1 and are further divided based on domain organisation into class IIa (HDACs 4, 5, 7, and 9) and class IIb (HDACs 6 and 10). The primary structure and organisation of the HDAC enzymes are shown, with amino acid length indicated at the right of the diagrams.

Contrary to the class I HDACs, the class II enzymes exhibit tissue-specificity, with expression in the heart, liver and/or kidney, and are involved with differentiation and developmental processes (Bannister and Kouzarides, 2011; Barneda-Zahonero and Parra, 2012). These are higher molecular weight molecules, between 120 kDa and 135 kDa (Egger *et al*., 2004; Laird, 2005; Mehnert and Kelly, 2007), and shuttle between the nucleus and cytoplasm, with subgroup IIa possessing a nuclear localisation signal while subgroup IIb lacks this domain (Khan and La Thangue, 2012). The negligible catalytic activity of the class II HDACs is likely due to the histidine substitution at their active site, rather than the tyrosine residue present in the class I HDACs. The class II enzymes have therefore been classified as 'pseudo-enzymes' and could function in non-enzymatic cellular roles (Lahm *et al*., 2007; Yang *et al*., 2008; Parra and Verdin, 2010; Barneda-Zahonero and Parra, 2012; Sun *et al*., 2013). It is also thought that any detectable deacetylase activity is due to their interaction with HDAC3 in co-repressor complexes (Fischel *et al*., 2002; Sun *et al*., 2013), discussed in further detail below. Despite this, these HDACs have a conserved catalytic deacetylase domain and an amino-terminal domain containing a regulatory serine residue that undergoes phosphorylation (Yang *et al*., 2008; Parra and Verdin, 2010; Barneda-Zahonero and Parra, 2012; Kelly and Cowley, 2013). HDACs 6 and 10 of subgroup IIb are unique in their structures in that they contain a second homologous deacetylase domain (Hubbert *et al*., 2002; Zou *et al*., 2006; Yang *et al*., 2013). Additionally, HDAC6 contains a carboxy-terminal ubiquitin-binding zinc finger domain and a nuclear export signal (Seigneur-Berny *et al*., 2001; Khan and La Thangue, 2012; Yang *et al*., 2013), and HDAC7 has an HDAC-specific zinc binding motif neighbouring the active site. It is likely that this domain functions in substrate recognition and protein-protein interactions (Schuetz *et al*., 2008).

The last Zn^{2+}-dependent class of HDACs is class IV, which contains only HDAC11. It shares conserved residues between both class I and II HDACs (Haberland *et al*., 2009; Barneda-Zahonero and Parra, 2012) and resides in the cytoplasm and nucleus (Kawaguchi *et al*., 2003; Liu *et al*., 2008; Yang and Seto, 2008; Khan and La Thangue, 2012). There has been little research into the function of HDAC11, but it is highly expressed in a tissue-specific manner, particularly in the kidney, brain and heart (Gao *et al*., 2002; Liu *et al*., 2008; Villagra *et al*., 2009).

Class III HDACs (SIRT 1 through SIRT 7) are structurally homologous to the yeast Sir2 protein and, as mentioned previously, require NAD^+ rather than Zn^{2+} for catalysis. Due to this difference in co-factor dependency, the class III HDACs are insensitive to HDI-mediated

inhibition (Dockmanovic *et al*., 2007) and will not be discussed in detail in this review.

1.3.3 Multi-Protein HDAC Co-Repressor Complexes

Class I and II HDACs cannot directly bind to DNA and are likely inactive without their activating partners. They exist in large multi-protein co-repressor complexes known as Sin3, NuRD (nucleosome remodelling and deacetylating), CoREST (co-repressor for element-1-silencing transcription factor), and NCoR/SMRT (nuclear receptor co-repressor/silencing mediator of retinoic and thyroid receptors), depending on the HDAC present (Zhang *et al*., 1999; Goodson *et al*., 2005; Ropero and Esteller, 2007; Perissi *et al*., 2010; Kelly and Cowley, 2013; Sun *et al*., 2013). HDACs 1 and 2 can form hetero- or homo-dimers (Luo *et al*., 2009; Delcuve *et al*., 2013) and are subunits within Sin3, NuRD and CoREST (Figure 1.2 A) (Yang and Seto 2008; Barneda-Zahonero and Parra, 2012), whereas HDAC3 dimerises with the class II HDACs and is found within the NCoR/SMRT complex (Figure 1.2 B) (Li *et al*., 2000). The co-repressor complex(es) associated with HDAC8 have yet to be identified (Barneda-Zahonero and Parra, 2012).

These multi-protein complexes are then targeted to chromatin by transcription factors such as p53, GATA4, E2F, pRb and/or STAT3 (Marks *et al*., 2004; Drummond *et al*., 2005; Lin *et al*., 2006; Liu *et al*., 2006; Mehnert and Kelly, 2007; Delcuve *et al*., 2013) and/or chromatin-altering enzymes (Zhang *et al*., 1999; Ropero and Esteller, 2007; Kelly and Cowley, 2013). In addition, methylated DNA can recruit HDAC complexes through methyl-binding proteins in order to repress gene expression (Jones *et al*., 1998; Nan *et al*., 1998; Ropero and Esteller, 2007). Interestingly, several nuclear receptors can form co-repressor complexes with HDACs in the absence of ligands, and can further influence gene expression (Lin *et al*., 2006).

These co-repressor complexes have diverse multi-protein components that dictate their functional activities (Table 1.2). The Sin3 co-repressor complex has deacetylase activity, and additional proteins associated with the Sin3 complex include SAP18, SAP30L, Ing2 and RbAp46/48 (Hayakawa *et al*., 2011; Hurst *et al*., 2012; Delcuve *et al*., 2013). The NuRD complex consists of protein components such as RbAp46/47, p66α/β, MBD2, MTA1/2/3 and LSD1. This co-repressor has lysine deacetylation and ATP-dependent helicase activities, carried out by HDAC1 and/or HDAC2 dimers and the Mi-2α/β helicase, respectively (Denslow *et al*.,

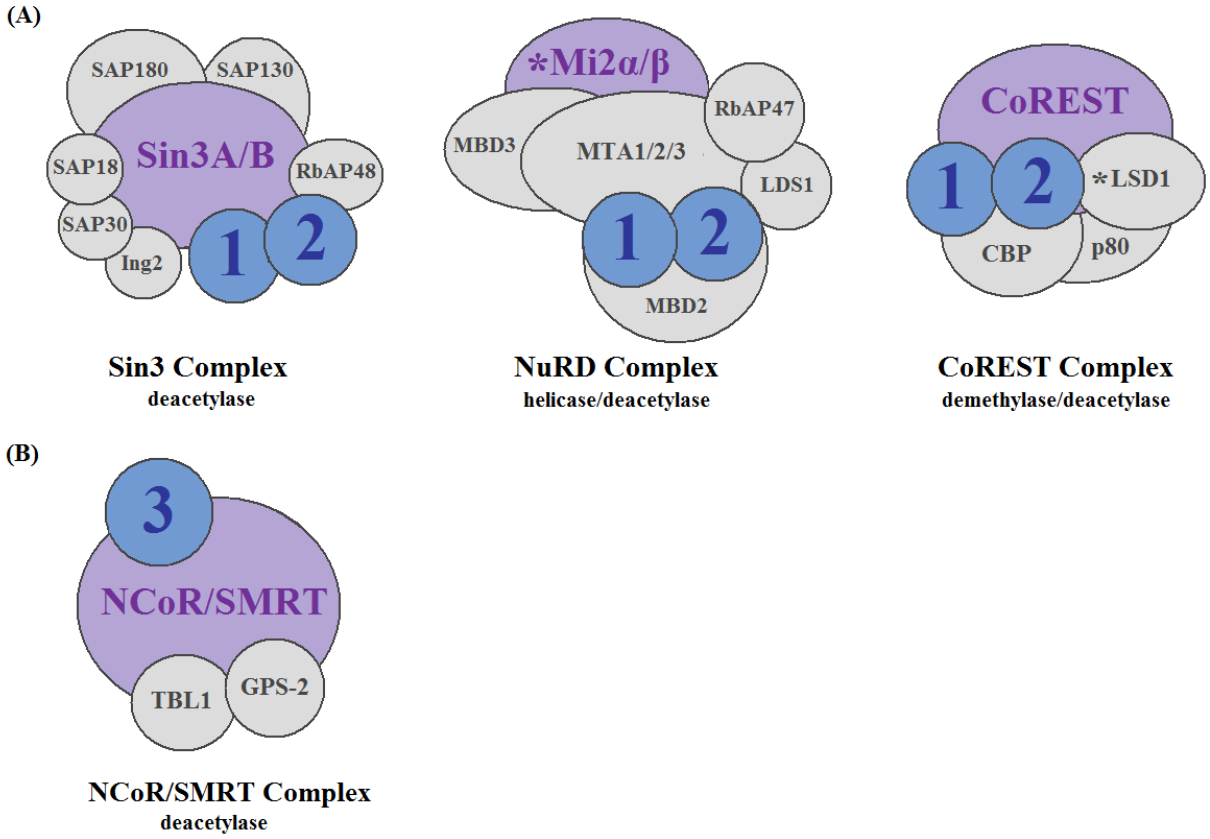

Figure 1.2. Schematic representation of histone deacetylases and their associated co-repressor complexes. (A) HDACs 1 and 2 form hetero- or homo-dimers within the Sin3, NuRD and CoREST complexes and (B) HDAC3 complexes with NCoR/SMRT. Shown are the various protein subunits currently known to form the complexes; the HDACs are indicated by their numbers in the blue colour and those associated with helicase or demethylation activity are denoted by an asterisk.

Table 1.2. The co-repressor complexes and their associated HDAC and protein subunits. HDAC 1 and 2 form hetero- or homo-dimers within the Sin3, NuRD and CoREST complexes, and HDAC3 forms a complex with NCoR/SMRT. The four co-repressor complexes have deacetylation activity while NuRD and CoREST also have helicase and demethylation activity, respectively.

Co-Repressor Complex	HDAC	Subunits	Function(s)
Sin3	1/2	SAP18 SAP30L RbAp46/48 Ing2	deacetylation
NuRD	1/2	RbAp46/47 p66α/β MBD2 MTA1/2/3 LSD1	deacetylation helicase
CoREST	1/2	LSD1 HMG20B PHF21A	deacetylation demethylation
NCoR/SMRT	3	TBL1 GPS-2	deacetylation

2007; Wang *et al*., 2009; Decluve *et al*., 2013). The CoREST complex has deacetylase and demethylase activity, and includes the LSD1, HMG20B and PHF21A proteins (Lakowski *et al*., 2006). The proteins associated with the HDAC3-containing complex NCoR/SMRT include TBL1 and GPS-2. This complex functions mainly in deacetylation (Li *et al*., 2000; Karagianni and Wong, 2007).

HDAC3 is unique in that it requires the deacetylase activating domain (DAD) of the NCoR/SMRT complex for enzymatic activity (Guenther *et al*., 2001; Sun *et al*., 2013), and this interaction is dependent upon an inositol tetraphosphate (IP_4) molecule (Watson *et al*., 2012). Due to the association of class II HDACs with HDAC3, and their deficient catalytic activity, it has recently been suggested that they potentially have a role in protein scaffolding rather than deacetylation (Schapira, 2011; Sun *et al*., 2013). In addition to the canonical co-repressor HDAC complexes, HDACs have also been found to be present in many other complexes. For example, SHMP is a complex consisting of HDAC1, Sin3B, MRG15 and PHF12, and is associated with the gene coding region during transcription (Jelinic *et al*., 2011; Delcuve *et al*., 2013).

1.3.3.1 HDAC Co-Repressor Regulation

The activities of HDAC-containing complexes can be regulated through subcellular localisation and post-translational modifications. Phosphorylation of HDAC1 at serines 393, 421 and 423, and HDAC2 serines 394, 422 and 424 are required to form co-repressor complexes (Tsai *et al*., 2002), and the acetylation of HDAC1 at lysine 432 inhibits the activity of HDAC1-containing dimers. For instance, the protein kinase CK2 has been found to be associated with the Sin3 and NuRD complexes and act to maintain the phosphorylation state of HDACs 1 and 2 for catalytic function (Luo *et al*., 2009; Spiegel *et al*., 2012; Delcuve *et al*., 2013). Additionally, the ubitiquination of HDACs 1 and 2 can lead to their degradation (Segre *et al*., 2011). Likewise, serine 424 phosphorylation on HDAC3 by CK2 stimulates its activity, while an additional level of regulation comes through its cytoplasmic or nuclear localisation mediated by the CRM1-pathway (Yang *et al*., 2002; Zhang *et al*., 2005; Karagianni and Wong, 2007; Yao and Yang, 2011). It has also been shown that protein phosphatase 4 (PP4) is associated with the NCoR/SMRT complex and contributes to HDAC3 regulation (Zhang *et al*., 2005).

In addition, the class II HDACs can be regulated through their subcellular localisation.

Phosphorylated HDACs 4 and 5 associate with the regulatory protein 14-3-3, which then blocks their nuclear translocation from the cytoplasm (Nishino *et al*., 2008). This prevents their binding to the HDAC3-containing co-repressor complexes and reduces their transcriptional activity (Grozinger *et al*., 1999; Nishino *et al*., 2008) In addition, protein phosphatase 2A (PP2A) can dephosphorylate HDAC4 and expose its nuclear localisation signal (Paroni *et al*., 2008; Nishino *et al*., 2008).

1.3.4 Physiological and Cellular Functions of HDACs

Diverse HDAC physiological and cellular functions (Table 1.3), as well as the importance of co-repressor binding, have been illustrated in knock-down and/or knock-out analyses in *Drosophila*, yeast and mice (Yang and Seto, 2008; Haberland *et al*., 2009; Kelly and Cowley, 2013). The focus of this section will be on mammalian HDACs. HDAC enzymes have been implicated in many cellular processes, including transcription, elongation, splicing, mRNA stability and mitosis (Lagger *et al*., 2002; Kelly and Cowley, 2013). The class I HDACs mainly function in cell survival and cellular growth. For instance, knocking out HDAC1 in cell lines negatively influences proliferation and up-regulates cyclin-dependent kinase inhibitors. Furthermore, despite high structural similarity between the enzymes, HDAC2 and HDAC3 could not rescue the impaired phenotype, highlighting their unique roles (Lagger *et al*., 2002).

The majority of *in vivo* research has centred on the developmental roles of the class I HDACs, particularly HDAC1 and HDAC2. Despite their high sequence similarity, murine models indicate that they possess non-redundant cellular functions that are critical for embryonic development and cardiovascular health (Haberland *et al*., 2009; Kelly and Cowley, 2013). The deletion of *hdac1* in mice results in embryonic lethality, and it is therefore thought to be involved in embryogenesis and cardiac development (Lagger *et al*., 2002; Zupkovitz *et al*., 2010), whereas conditional knock-out of both *hdac1* and *hdac2* alleles impairs neural tissue development (Montgomery *et al*., 2009; Chen *et al*., 2011). In addition, histones H3 and H4 were hypo-acetylated in *hdac*-null embryos, and *hdac1* deletion in embryonic stem cells enhances expression of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ (Lagger *et al*., 2002). Furthermore, deletion of HDAC3 is embryonically lethal in mouse models (Bhaskara *et al*., 2008; Khan and La Thangue, 2012).

Table 1.3. The cellular and physiological functions of HDACs. Indicated are select cellular and physiological functions for the class I (HDACs 1, 2, 3 and 8), class IIa (HDACs 4, 5, 7 and 9), class IIb (HDACs 6 and 10) and the class IV (HDAC11). Further listed are the knock-out phenotypes observed in mouse studies.

	HDAC	Cellular Function(s)	Physiological Function(s)	Knock-Out Phenotype
Class I	1	gene activation	embryogenesis cardiogenesis	embryonic lethality impaired neural tissue development
	2	gene activation	embryogenesis cardiogenesis	impaired neural tissue development cardiac abnormalities
	3	cell cycle arrest DNA repair mitosis apoptosis	--	embryonic lethality
	8	--	--	--
Class IIa	4	gene expression	cardiogenesis	--
	5		angiogenesis	--
	7	apoptosis	angiogenesis	--
	9	angiogenesis	--	angiogenic abnormalities cardiac abnormalities
Class IIb	6	cellular growth apoptosis chaperone autophagy protein degradation	angiogenesis	--
	10	autophagy	--	--
Class IV	11	--	--	--

Knock-out studies in mice have also illustrated that HDACs 1 and 2 can regulate gene expression in both a positive and negative manner (Zupkovitz *et al*., 2006; Yamaguchi *et al*., 2010; Kelly and Cowley, 2013). Chromatin immuno-precipitation (ChIP) partnered with high-throughput sequencing (ChIP-Seq) indicated that these HDACs associate with transcriptionally active genomic regions (Wang *et al*., 2009). It is speculated that this counteracts the activity of HATs and RNA pol II in a dynamic, cyclic process of gene inactivation and activation (Kelly and Cowley, 2013). Silencing and/or knock-down of *hdac1* and *hdac2* in cell lines demonstrate reduced proliferation and increased expression of $p21^{WAF1}$ *in vitro* (Wilting *et al*., 2010; Yamaguchi *et al*., 2010; Zupkovitz *et al*., 2010; Kelly and Cowley, 2013).

In addition, knocking down expression of HDAC3 in cells leads to cell cycle arrest, impaired DNA repair responses and apoptotic dysregulation (Bhaskara *et al*., 2008; Barneda-Zahonero and Parra, 2012; Khan and La Thangue, 2012; Reichert *et al*., 2012). As previously mentioned, HDAC3 requires the DAD domain of NCoR/SMRT and an IP4 molecule for its deacetylation activity. Interestingly, mouse models with knock-in mutations that obliterate these interactions are able to live to adulthood. Mutational analysis has also demonstated that HDAC3 deacetylase activity is not required; this suggests that the NCoR/SMRT and HDAC3 complex also functions independently of its deacetylation activity (Sun *et al*., 2013; You *et al*., 2013).

While the majority of research has investigated the function of class I HDACs, murine models indicate that class II HDACs are involved in muscle and neuronal differentiation, adipogenic gene regulation and angiogenic processes (Nebbioso *et al*., 2010). HDAC9 knock-out in transgenic mice can lead to increased cardiac growth (Zhang *et al*., 2002) and angiogenic abnormalities, such as decreased retina vascularisation and reduced blood flow (Kaluza *et al*., 2013). It has further been illustrated that HDAC4 through HDAC7 are negative regulators of angiogenic processes (Vega *et al*., 2004; Urbich *et al*., 2009; Khan and La Thangue, 2012; Kaluza *et al*., 2013), while HDAC7 can also negatively regulate apoptosis (Dequiedt *et al*., 2003). Furthermore, through their interaction with the PPARγ signalling pathway, the class II HDACs have been implicated in heart and adipose tissue development *in vivo* (Nebbioso *et al*., 2010). Interestingly, both HDAC2- and HDAC9-deficient mice present with cardiac abnormalities, although via different signalling pathways (Zhang *et al*., 2005; Trivedi *et al*., 2007).

Through its ubiquitin-binding zinc finger domain, HDAC6 functions in numerous cellular processes, including proliferation, apoptosis, chaperone functions, autophagy, and the ubiquitin-proteosomal and misfolded protein degradation systems. These functions are likely achieved through interaction with ubiquitin mediated by the ubiquitin-binding domain (Rubinsztein *et al*., 2006; Olzmann *et al*., 2007; Lee *et al*., 2010; Peng and Seto, 2011; Yang *et al*., 2013). In addition to cancer, HDAC6 has also been implicated in many neurological and protein conformational disorders, many of which harbour abnormal pathways such as protein degradation and autophagy (Yang *et al*., 2013).

Histone deacetylases have many physiological roles, and while the activities of some enzymes are redundant, the importance of HDAC activity in the organism is highlighted by *in vivo* knock-out and/or knock-down studies (Yang *et al*., 2008; Haberland *et al*., 2009; Kelly and Cowley, 2013). It is also interesting to note that HDACs function independently of their deacetylase activity, further highlighting their complicated roles in transcription and cellular homeostasis (Sun *et al*., 2013; You *et al*., 2013). Contributing to their complicated and robust nature is their ability to deacetylate non-histone proteins (Kim *et al*., 2006; Choudhary *et al*., 2009), prompting a movement in the literature to renaming them as 'lysine deacetylases' (KDACs). While histones were considered the central substrates for these enzymes, their non-histone targets and activities are seemingly just as vital (Gregoretti *et al*., 2004; Dockmanovic *et al*., 2007; Xu *et al*., 2007).

1.3.5 Non-Histone Targets of HDACs

Phylogenetic analysis has demonstrated that HDAC enzymes predate histone proteins (Gregoretti *et al*., 2004) and are conserved in many species (Yu *et al*., 2008; Zhang *et al*., 2009; Wang *et al*., 2010), hence, it is unsurprising that there has been increasing evidence that they target non-histone proteins. Protein acetylation is one of the most common post-translational modifications which affect protein regulation. As previously indicated, the first acetylated proteins identified were histones and subsequently twenty-seven years later, the responsible enzymes were discovered and named after their common substrates (Inoue and Fujimoto, 1969; Taunton *et al*., 1996; Yao and Yang, 2011). There are approximately 1700 proteins which can be acetylated and deacetylated by HDACs. These include cytoplasmic proteins and nuclear

transcription factors, such as p53, GATA4, ERα, E2F1 and NF-κB (Kim *et al*., 2006; Choudhary *et al*., 2009; Peng and Seto, 2011; Kelly and Cowley, 2013).

The acetylation of lysines offers an additional layer of protein regulation and affects protein stability, cellular localisation, and protein-DNA or protein-protein interactions (Singh *et al*., 2010). While the deacetylation of transcription factors can indirectly influence transcriptional regulation, the deacetylation of non-histone proteins can exert physiological effects that are independent to transcription. These include apoptosis, cell cycle progression, autophagy, cell motility, chaperone functions and differentiation. Many of these biological processes can be controlled via HDACs in both non-transcriptional and transcriptional manners (Ropero and Esteller, 2007; Kaluza *et al*., 2013), and certain processes are highlighted in further detail.

The first non-histone target identified for HDAC enzymes was the p53 tumour suppressor protein (Gu and Roeder, 1997). It contains many potential lysine sites that can be targeted for acetylation by at least HDACs 1, 2 and 3 (Juan *et al*., 2000; Wang *et al*., 2003; Luo *et al*., 2004; Zeng *et al*., 2006; Karagianni and Wong, 2007; Ropero and Esteller, 2007). Following DNA damage, p53 is stabilised through acetylation by the p300/CBP acetyltransferase, thereby increasing its DNA binding capability and activating p53-responsive target genes. This ultimately leads to cell cycle arrest and apoptosis. Thus, HDAC-mediated deacetylation decreases p53 protein stability and diminishes DNA binding, resulting in cell cycle arrest and apoptotic inhibition (Juan *et al*., 2000; Luo *et al*., 2000). For instance, it has been illustrated in MCF-7 breast cancer-derived cell lines that HDAC2 functions in the p53 pathway. Silencing HDAC2 induced p53-dependent and p53-independent senescence and cell cycle arrest at the G1 phase. HDAC2 negatively regulates p53-dependent transcriptional activity, and knocking down HDAC2 increases p53-binding to promoters. Furthermore, MYC expression was also down-regulated in a p53-dependent manner (Harms and Chen, 2007).

STAT1 deacetylation prevents the phosphorylation required for its nuclear translocation and DNA binding (Kramer *et al*., 2009). In addition, the STAT1 transcription factor is deacetylated to destabilise its homo- and hetero-dimerisation ability (Yuan *et al*., 2005). Despite their classification of pseudo-enzymes (Fischel *et al*., 2002; Lahm *et al*., 2007; Yang *et al*., 2008; Parra and Verdin, 2010; Sun *et al*., 2013), class II HDAC4 and HDAC7 can deacetylate p53, RUNX (runt domain transcription factor)-2 and HIF-1α (hypoxia inducible factor 1α). However, this is likely due to their association with HDAC3 (Yao and Yang, 2011). It is also interesting to

note that HDAC3 is recruited to and associated with chromosomes during mitotic division (Li *et al*., 2006; Karagianni and Wong, 2007). Furthermore, HDAC3 is critical for the kinetochore-microtubule formation and the assembly of mitotic spindles (Ishii *et al*., 2008), and is required to deacetylate histone H3 lysine 4 (H3K4) during sister chromatid attachment (Eot-Houllier *et al*., 2008). It is thought that the mitotic defects mediated by HDAC3 are independent of histone acetylation. In *Drosophila*, it has been shown that the centromere has a high concentration of tri-methylated histone H3 lysine 9 ($H3K9me^3$), likely due to HDAC3 inhibition and its non-acetylated functions (Warrener *et al*., 2010).

In an additional non-transcriptional mechanism, the deacetylation of the apoptotic regulator Ku70 interferes with its ability to bind BAX, thus inhibiting apoptosis (Yamaguchi *et al*., 2009). While SIRT1, a class III HDAC, is thought to be the main deacetylase responsible (Cohen *et al*., 2004), HDACs belonging to class I can also act upon Ku70 (Subramanian *et al*., 2005). In addition, MEF2 (myocyte enhancer factor 2) interacts with HDAC4 and indirectly with HDAC3. Accordingly, HDAC4 is expressed highly in muscle tissue and may be involved in myogenesis through its interaction with MEF2. The deacetylation of MEF2 by HDAC3/HDAC4 represses the myoD gene promoter (Grozinger *et al*., 1999; Gregoire *et al*., 2007).

HDAC6 plays a role in the regulation of autophagy through promoting autophagosome and lysosome fusion by assembling F-actin fibres (Lee *et al*., 2010). In addition, HDAC6 can regulate cell adhesion, motility and protein chaperone functions (Hubbert *et al*., 2002; Wang *et al*., 2010) through targeting α-tubulin (Yang *et al*., 2008; Barneda-Zahonero and Parra, 2012), heat shock protein 90 (Hsp90) and cortactin for deacetylation (Valenzuela-Fernandez *et al*., 2008; Yang *et al*., 2013). For example, deacetylation of the chaperone protein Hsp90 at lysine 294 can decrease its function and interaction with client proteins such as the glucocorticoid receptor (Kovacs *et al*., 2005; Scroggins *et al*., 2007).

Many additional non-histone targets of HDACs have also been identified. Briefly, GATA2 interacts with HDAC3 to suppress transcription mediated by this transcription factor (Osawa *et al*., 2001), and HDAC3 can also deacetylate SRY, thus leading to the loss of its nuclear localisation (Thevenet *et al*., 2004). HDACs 1 and 3 deacetylate the p65 subunit of NF-κB, which enhances its DNA binding affinity (Kiernan *et al*., 2003; Singh *et al*., 2010).

While the possible roles of these HDACs in carcinogenesis are detailed in the following section, it is interesting to note that oestrogen increases the protein and mRNA expression of

HDAC6 and results in enhanced cell motility, contributing to metastatic processes. It has been shown that the anti-oestrogen tamoxifen decreases tubulin deacetylation mediated by HDAC6, thus reducing motility and metastatic potential (Saji *et al*., 2005). It has also been illustrated that HDAC1 can reduce the activity of oestrogen receptor-alpha (ERα) signalling (Kawai *et al*., 2003).

1.3.6 Role of HDACs in Tumorigenesis

The over-expression, dysregulation and/or mutation of HDACs, with subsequent aberrant genomic acetylation, has been implicated in many cancers (Sjoblom *et al*., 2006; Ma *et al*., 2009; Wanczyk *et al*., 2011; Barneda-Zahonero and Parra, 2012; Kelly and Cowley, 2013; Timp and Feinberg, 2013). For example, the class IIa HDAC4 has been found mutated in breast cancers (Sjoblom *et al*., 2006; Timp and Feinberg, 2013), and truncating or inactivating mutations have been observed in 4% of these cancer types (Ropero *et al*., 2006).

It has also been observed that HDACs can aberrantly interact with oncogenic fusion proteins that result from chromosomal translocations linked to some haematological cancers (Barneda-Zahonero and Parra, 2012; Ropero and Esteller, 2007). In particular, the fusion proteins RARα-PML, RARα-PLZF and AML-ETO, have the ability to recruit HDACs through their association with retinoic acid-responsiveness elements (RAREs) in gene promoters. This results in disruption of the cell cycle and abberant growth (Lin *et al*., 2001; Mehnert and Kelly, 2007; Ropero and Esteller, 2007). Therefore, there has been extensive research through siRNA knock-down and/or over-expression studies into the role of HDACs in tumorigenesis.

The class I HDACs are frequently dysregulated in malignancies; the enhanced expression of HDAC1 is associated with poorer prognosis and has been observed in diverse cancers, including gastric, breast, renal, colorectal, pancreatic, prostate and lung carcinomas (Choi *et al*., 2001; Zhang *et al*., 2005; Fritzsche *et al*., 2008; Weichert *et al*., 2008; Wilson *et al*., 2008; Adams *et al*., 2010; Minamiya *et al*., 2011). Similarly, HDAC2 over-expression has been observed in cervical, gastic and colorectal cancers (Song *et al*., 2005; Zhu *et al*., 2005; Ropero and Esteller, 2007). In breast cancer, HDAC1 and HDAC3 up-regulation correlate with expression and signalling of PgR and ERα hormone receptors (Krusche *et al*., 2005; Zhang *et al*., 2005; Barneda-Zahonero and Parra, 2012). Additional studies have illustrated that expression of

HDAC8 is increased in many childhood neuroblastomas (Oehme *et al*., 2009).

As previously mentioned, the class I HDACs are associated mainly with cell proliferation (Glaser *et al*., 2003; Weichert *et al*., 2008; Barneda-Zahonero and Parra, 2012). For example, in breast cancer cell lines, knocking down HDAC1 suppressed proliferation and increased apoptosis (Senese *et al*., 2007), whereas silencing HDAC2 lead to increased DNA-binding activity of the tumour suppressor p53, as well as cellular senescence and cell cycle arrest (Harms and Chen, 2007). The knock-down of HDAC2 and HDAC8 increased apoptosis and differentiation, and decreased proliferation in cervical cancer cells (Barneda-Zahonero and Parra, 2012; Hua *et al*., 2014), as well as inhibited proliferation in lung, colon and cervical cancer cell lines, respectively (Vannini *et al*., 2004; Barneda-Zahonero and Parra, 2012). Furthermore, it was illustrated that up-regulation of HDACs 1 and 8, as well as the class IIb HDAC6, increased invasion of breast cancer cells (Park *et al*., 2011). In addition, the ubiquitination of HDAC6 has been shown to be involved in tumorigenesis, wherein it interferes with normal gene expression during chromatin condensation (Hideshima *et al*., 2005; Yang *et al*., 2013).

While extensive data have implicated class I HDAC involvement in tumorigenesis, the class II HDACs are likewise frequently up-regulated in cancer and associated with poor survival (Barneda-Zahonero and Parra, 2012; Timp and Feinberg, 2013). For instance, Waldenstrom's macroglobulinaemia can be associated with amplification of HDACs 4 and 9 (Sun *et al*., 2011), and expression of HDACs 5 and 9 are increased in certain medulloblastomas. In these cases, the silencing of HDACs 5 and 9 has lead to apoptosis (Milde *et al*., 2010). The over-expression of HDAC9 has also been observed in cervical cancer (Choi *et al*., 2007). In advanced-stage oral squamous cell carcinoma, expression of HDAC6 was significantly higher than normal tissue or early-stage cancers (Sakuma *et al*., 2006); due to the role of HDAC6 in cell motility and adhesion, high levels can lead to decreased actin acetylation, which can thus increase migration of cancer cells and metastatic potential (Hubbard *et al*., 2002; Wang *et al*., 2010). Furthermore, HDAC6-mediated deacetylation of cortactin can increase its association with F-actin, enhancing cell motility (Zhang *et al*., 2007). In breast cancer, HDAC7 up-regulation leads to ERα-dependent cellular growth (Malik *et al*., 2010). In addition to mutations in HDAC4 (Sjoblom *et al*., 2006; Timp and Feinberg, 2013), its protein expression can also be increased in breast cancer (Ozdag *et al*., 2006) and it is thought to have a role in the repression of the cell cycle inhibitor $p21^{WAF1}$ through interaction with the Sp1 transcription factor (Mottet *et al*., 2009).

It has been established that the dysregulation of differentiation is a hallmark of cancer (Peng and Seto, 2011). While extensive research has highlighted direct transcriptional-mediated impacts on differentiation, non-transcriptional-mediated processes can also affect differentiation. For instance, HDAC1 has been shown to act upon the myogenic activator (myoD), decreasing its transcriptional activity (Mal *et al*., 2001; Peng and Seto, 2011). Additionally, the myocyte enhancer factor 2 (MEF2) transcription factor can be acted upon by HDACs 3 and 4, although the extent of HDAC4 involvement is currently unknown. Together, these contribute to tumorigenesis through enhanced proliferation (Zhoa *et al*., 2005; Gregoire *et al*., 2007). The acetylation status of RUNX1 is reversed by HDACs 4 and 5, and this abrogates its transformative ability (Jin *et al*., 2004; Yamaguchi *et al*., 2009). Additionally, under hypoxic conditions, the protein stability and activity of the HIF-1α transcription factor can be positively influenced through binding to HDACs 1 and 3. In this manner, HDACs can influence HIF-1α-mediated pro-angiogenic processes (Kim *et al*., 2007).

Despite the plethora of research implicating HDAC over-expression in carcinogenesis, contradictory observations have also been recorded. While high levels of HDAC1 have been established in many cancers (Minamiya *et al*., 2011), it had also been observed that over-expression correlated with improved survival in ERα- and PgR-positive breast cancers (Krusche *et al*., 2005; Sudo *et al*., 2011; Theocharis *et al*., 2011). Genetic mutations and loss-of-function in HDAC1 enzymatic activity enhanced expression of genes controlled by the retinoblastoma (Rb) protein, a negative regulator of the cell cycle, potentially leading to tumorigenesis (Frolov and Dyson, 2004; Ropero and Esteller, 2007). In addition, knocking down HDAC1 and HDAC2 leads to leucaemia, while silenced HDAC3 is associated with hepatocellular carcinoma (Bhaskara *et al*., 2010; Santoro *et al*., 2013; Sun *et al*., 2013). Furthermore, NCoR/SMRT up-regulation suppressed breast cancer progression through reduced signalling via the androgen receptor (Qi *et al*., 2013).

A dual role for HDAC2 in neoplasia has also been reported; loss-of-function mutations have been observed in sporadic and hereditary non-polyposis colorectal cancer. The mechanism of tumorigenesis has yet to be elucidated, but the mutation reduces HDAC2 protein expression and activity (Ropero *et al*., 2006; Ropero and Esteller, 2007). In addition, HDAC6 is up-regulated in breast cancer (Zhang *et al*., 2004; Ropero and Esteller, 2007; Timp and Feinberg, 2013) and can be associated with improved prognosis (Zhang *et al*., 2004); however a second

study of breast cancer tissue illustrated no prognostic benefit regarding HDAC6 expression (Saji *et al*., 2005; Barneda-Zahonero and Parra, 2012). Interestingly, HDAC4 can negatively or positively regulate $p21^{WAF1}$; upon DNA damaging agents, p53 recruits HDAC4 to increase $p21^{WAF1}$ expression and inhibit cell cycle progression (Mottet *et al*., 2009).

1.3.7 Summary

The dysregulation of HDACs has been implicated in cancer, contributing to aberrant cellular homeostasis contributing to tumorigenesis. HDACs are involved in many cellular pathways and are quite complex in their functions, and it has become increasingly important to understand how HDACs work and are dysregulated in cancer (Peng and Seto, 2011). While HDAC involvement in cancers has been controversial, with some exhibiting properties of tumour suppressor genes in rare cases, there has been extensive evidence that abnormal HDAC activity contributes to carcinogenesis (Choi *et al*., 2001; Zhang *et al*., 2005; Sjoblom *et al*., 2006; Fritzsche *et al*., 2008; Weichert *et al*., 2008; Ma *et al*., 2009; Minamiya *et al*., 2011; Wanczyk *et al*., 2011; Timp and Feinberg, 2013). Thus, targeting HDACs have been considered a viable therapeutic option, and there are many synthetic and natural compounds in pre-clinical or clinical trials that inhibit these enzymes. These HDAC inhibitors have been shown to be pro-apoptotic to cancer cells and several have shown promising results in clinical trials (Piccart-Gebhart *et al*., 2005; Piekarz *et al*., 2007; Bicaku *et al*., 2008; Fan *et al*., 2008; Lee *et al*., 2008; Chavan and Somani, 2010; Martinet and Bertrand, 2011; Perez *et al*., 2011; Slamon *et al*., 2011; Lamond and Younis, 2014; Li and Zhu, 2014).

1.4 Histone Deacetylase Inhibitors

The first natural compound identified to have inhibitory activity on HDAC enzymes was butyrate. This is a short chain fatty acid produced in the colon by the anaerobic bacterial fermentation of dietary fibre. It has been shown to prevent colon carcinogenesis in part through inhibiting DNA synthesis, proliferation and altering gene expression at the millimolar concentration (Canido *et al*., 1978; Hinnebusch *et al*., 2002; Davie *et al*., 2003). It was later observed that this activity was in part due to the ability of butyrate to inhibit HDACs, thus

identifying it as the first 'pan-specific' histone deacetylase inhibitor (HDI). Further supporting this, butyrate has been shown to increase acetylation of histones H3 and H4 *in vitro* and *in vivo* (Boffa *et al*., 1978; Canido *et al*., 1978; Hinnebusch *et al*., 2002; Davie *et al*., 2003).

These HDIs are a novel class of chemotherapeutic and chemopreventative agents that act in part by inhibiting the activity of HDAC enzymes to induce global protein acetylation via the activity of histone acetyltransferases. Partially through up-regulating tumour suppressor genes and/or down-regulating oncogenes, HDI treatment selectively induces apoptosis, differentiation and cell cycle arrest in tumour cells (Mehnert *et al*., 2007; Ma *et al*., 2009; Nebbioso *et al*., 2010; Wanczyk *et al*., 2011). In addition to acetylation of histones and alterations in gene expression, HDIs also induce acetylation of non-histone proteins (Kim *et al*., 2006; Harms and Chen, 2007; Choudhary *et al*., 2009; Peng and Seto, 2011; Kaluza *et al*., 2013; Kelly and Cowley, 2013) and have been shown have genome-wide anti-neoplastic consequences. Briefly, HDI treatment alters the acetylation of transcription factors (Ito *et al*., 2002), induce polyploidy (Xu *et al*., 2005), cause mitotic slippage during mitosis (Stevens *et al*., 2008) and premature chromatid separation (Magnaghi-Jaulin *et al*., 2007), and disrupt HDAC-protein phosphatase complexes (Chen *et al*., 2008). Certain aspects of these processes will be discussed in greater detail below.

1.4.1 Cellular Actions of HDIs

It has been demonstrated that HDAC inhibition increases the accumulation of H3K9/14 acetylation, which in turn triggers the methylation of H3K4 at gene promoters, thus activating transcription (Felsenfeld, 1992; Wolffe, 1994; Yang *et al*., 2000; Keen *et al*., 2003; Jang *et al*., 2004; Sharma *et al*., 2006; Ueda *et al*., 2006; Ropero and Esteller, 2007; Tsai and Baylin, 2011; Stark *et al*., 2013; Marmorstein and Zhou, 2014). Despite the established role of HDACs in transcriptional activation, inhibiting HDACs through HDI treatment only results in the up-regulation of a small subset of genes, approximately 2-10% depending on the study and methods utilised. It has also been observed that a similar number of genes are repressed following HDI administration (Van Lint *et al*., 1996; Gray *et al*., 2004; Marks *et al*., 2004; Mitsiades *et al*., 2004; Peart *et al*., 2005; Ropero and Esteller, 2007; LeBonte *et al*., 2009). While HDI-mediated histone acetylation is partially responsible for gene up-regulation, the down-regulation of gene expression cannot be explained by enhanced histone acetylation (Ellis *et al*., 2008). In addition,

while some HDIs can regulate highly similar genes in a cell line and result in apoptosis or cell cycle arrest, there are differences between other HDI treatments and altered gene expression profiles. For instance, while HDIs collectively lead to apoptotic phenotypes, the gene expression profiles associated with each drug are different (Mitsiades *et al*., 2004; Peart *et al*., 2005). Therefore, the effects of HDIs on cellular processes are pleiotrophic and often cell-specific. It has been established that HDIs can affect the acetylation of transcription factors and cytoplasmic proteins, playing a direct or indirect role in apoptosis, angiogenesis, cell cycle progression, mitotic cell death, and/or autophagy (Khan and La Thangue, 2012). These effects are summarised in Figure 1.3.

While the induction of genes, particularly the commonly-studied $p21^{WAF1}$, by HDIs has been extensively studied, the effects of these drugs upon transcription will be the focus of a forthcoming section and will not be discussed below in great detail.

1.4.1.1 Acetylation of Non-Histone Proteins

As previously discussed, a vast majority of non-histone targets have been identified for HDACs (Gu and Roeder, 1997; Kim *et al*., 2006; Choudhary *et al*., 2009; Singh *et al*., 2010; Peng and Seto, 2011; Kelly and Cowley, 2013), and HDIs affect the acetylation pattern of many cytosolic and nuclear proteins (Ito *et al*., 2002). These can influence numerous cellular processes, such as the ubiquitin-proteosome system, the cell cycle and apoptosis (Hubbard *et al*., 2002; Wang *et al*., 2010). In addition, HDIs can decrease or increase the stability and/or alter the activity of transcription factors; in this manner, HDIs can indirectly influence gene expression (Luo *et al*., 2000; Vigushin *et al*., 2001; Bicaku *et al*., 2008; Ma *et al*., 2009; Chatterjee *et al*., 2013). For example, the transcription factor p53 is acetylated and stabilised following HDI treatment (Luo *et al*., 2000; Ma *et al*., 2009). The acetylation of Hsp90 (Kovacs *et al*., 2005) and ERα (Vigushin *et al*., 2001; Bicaku *et al*., 2008) are also enhanced after treatment with HDIs and effect downstream cellular processes.

Particularly, HDAC6 has been shown to have a major role in the ubiquitin-proteasome system and the misfolded protein response (Kawaguchi *et al*., 2003; Boyault *et al*., 2006). The chaperone protein Hsp90 is targeted by HDAC6 for deacetylation, and class II-specific or 'pan-inhibitory' HDIs hyper-acetylate Hsp90 through inhibiting HDAC6 activity, thus abrogating its

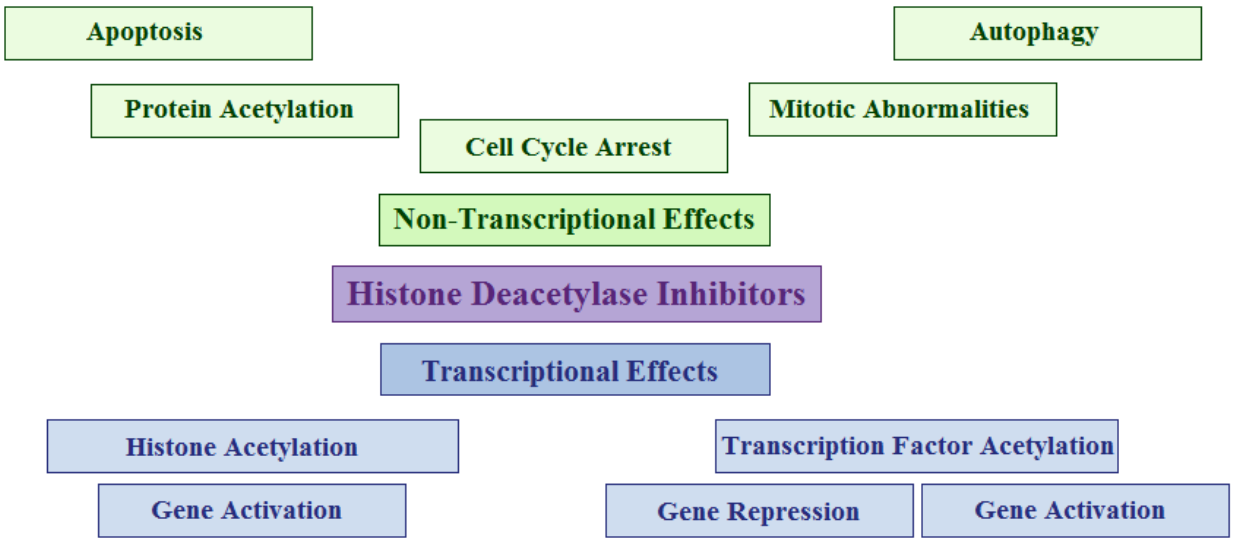

Figure 1.3. Pleiotrophic effects of histone deacetylase inhibitors. Included are the non-transcriptional and transcriptional effects of the class of inhibitors. Cellular pathways activated upon treatment include apoptosis, cell cycle arrest, autophagy, mitotic abnormalities, and histone or non-histone protein acetylation leading to altered gene regulation.

chaperone function. This ultimately leads to the degradation of its oncogenic substrates, such as Akt (Rao *et al.*, 2008; Khan and La Thangue, 2012). The inhibition of HDAC6 also results in the accumulation of acetylated tubulin, stabilising the molecule and potentially inhibiting tumour cell growth (Glaser *et al.*, 2003; Khan *et al.*, 2008; Yasumichi *et al.*, 2010).

1.4.1.2 Inhibition of the Cell Cycle

It is generally recognised that a global effect of HDI treatments are their shared ability to inhibit progression through the cell cycle (Sakajiri *et al.*, 2005; Komatsu *et al.*, 2006; Ma *et al.*, 2009). While the inhibition of the cell cycle at G2/M affects both non-transformed and transformed cells, the latter generally lack a functioning G2 checkpoint and thus undergo apoptosis (Zhao *et al.*, 2005; Zupkovitz *et al.*, 2010). The arrest of cellular growth at the G1/S or G2/M phase is partly due to the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ (Yoshida *et al.*, 1995; Ogryzko *et al.*, 2996; Noh *et al.*, 2003; Gui *et al.*, 2004). Vorinostat mediates cell cycle arrest through two potential mechanisms; by disrupting the function of the Sin3 repressor complex mediated via Ing2 dissociation and by direct inhibition of the HDAC enzymes (Sardui *et al.*, 2014). It has been further demonstrated that Vorinostat interferes with Sin3 binding to the $p21^{WAF1}$ promoter through this mechanism (Smith *et al.*, 2010).

In addition, the retinoblastoma protein (pRb), a negative regulator of the cell cycle, down-regulates E2F-dependent gene expression through the recruitment of chromatin modifying enzymes to gene promoters. pRb binds to class I HDACs and Sin3 complex, thus altering the balance of histone acetylation and repressing gene expression. In addition, HDACs negatively affect transcription of E2F-dependent genes in G1 phase (Sellers *et al.*, 1995; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Meloni *et al.*, 1999; Ross *et al.*, 2001; Rayman *et al.*, 2002; Frolov and Dyson, 2004). It has been demonstrated that TSA impedes pRb-mediated inhibition of E2F-dependent genes (Zhang *et al.*, 2000). In addition to the induction of cyclin-dependent kinase inhibitors, the cyclin family of proteins are repressed following HDI treatment (Mateo *et al.*, 2009; Jin *et al.*, 2012; Zhang *et al.*, 2012; Vidal-Laliena *et al.*, 2013), leading to a reduction in active, phosphorylated pRb, subsequent downstream signalling and cell cycle progression (Zhao *et al.*, 2005; Wu and Yu, 2009). Furthermore, HDIs stimulate degradation of the G1/S phase regulator cyclin D1, in an ubiquitin-26S dependent pathway (Alao *et al.*, 2006).

1.4.1.3 Pro-Apoptotic Effects

HDI-mediated acetylation of non-histone proteins favours apoptosis (Insinga *et al*., 2005; Bolden *et al*., 2006). The mechanisms of cell death are myriad and include the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways, and reactive oxygen species (ROS)-mediated cell death. In addition, apoptosis is the end-point of various HDI-mediated defects in DNA repair mechanisms and mitotic abnormalities (Insinga *et al*., 2005; Bolden *et al*., 2006; Mehnert and Kelly, 2007; Khan and La Thangue, 2012). For example, it has been observed that HDI-treated cells possess damaged pericentric heterochromatin and impaired segregation of chromosomes during mitosis. Disrupting mitotic division initiates apoptosis and mitotic slippage, a phenomenon wherein mitotic cells re-enter G1 phase without completing mitosis (Gabrielli and Brown, 2012). In addition, HDIs can also inhibit expression of proteins responsible for the spindle assembly checkpoint, leading to cell death (Taddei *et al*., 2001; Inche *et al*., 2006; Li *et al*., 2006; Stevens *et al*., 2008).

HDI treatment results in a gene induction signature generally associated with pro-apoptotic events. The up-regulation of death receptors and ligands leads to apoptosis in transformed cells, as well as in murine models (Nebbioso *et al*., 2005; Borbone *et al*., 2010; Khan and La Thangue, 2012). In addition, induction of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) ultimately leads to activation of caspase 3 via the extrinsic pathway (Nebbioso *et al*., 2005; Borbone *et al*., 2010; Srivastava *et al*., 2010). HDIs also down-regulate pro-survival proteins and up-regulate pro-apoptotic proteins, such as Bcl-2 and BAX, leading to cellular death through the intrinsic pathway (Rikiishi *et al*., 2011). In fact, HDI-mediated apoptosis had been abrogated in cell lines with reduced levels of the BAX protein (Ierano *et al*., 2013). Additionally, the acetylation of the chaperone protein Hsp90 leads to its destabilisation and subsequent degradation of its oncogenic substrates (Yu *et al*., 2002; Mehnert and Kelly, 2007; Kramer *et al*., 2014). Furthermore, ROS are a by-product of caspase-independent apoptosis, and HDI treatment increases ROS concentration within the cell, further exacerbating HDI-mediated cell death (Ungerstedt *et al*., 2005; Mehnert and Kelly, 2007; Bhalla *et al*., 2009).

It has also been established that HDIs lead to apoptosis through activating the oncogenic pRb-E2F pathway (Zhao *et al*., 2005). Interestingly, defects in the pRb-E2F pathway are able to increase proliferative genes, as well as pro-apoptotic genes (Sherr *et al*., 2002). Drug treatment

increases the association of the transcription factor E2F1 with the promoter of the pro-apoptotic BH3-only protein BIM. Due to this, cells with elevated E2F1 expression are more susceptible to HDI-mediated apoptosis (Zhao *et al.*, 2005).

1.4.1.4 Additional HDI-Mediated Anti-Neoplastic Effects

As previously indicated, HDI administration activates numerous anti-tumorigenic pathways. For instance, HDIs mediate gene signatures associated with DNA repair, ultimately leading to cell death (Bakkenist *et al.*, 2003; Adimoolam *et al.*, 2007; Zhang *et al.*, 2007). In addition, the autophagic-lysosomal fusion degradation process is induced in cancer cells treated with these drugs (Rikiishi *et al.*, 2011). HDI treatment also inhibits metastasis through up-regulation of certain metastatic suppressors (Ma *et al.*, 2009). The inhibition of class II HDACs can lead to cell-specific and retinoic acid-dependent differentiation in mouse embryonic carcinoma cells (Slingerland *et al.*, 2013). Furthermore, HDIs have the ability to decrease the action of HDACs 1, 2 and 3 on HIF-1α, inhibiting angiogenesis (Kim *et al.*, 2007). Therefore, it has been well established that HDIs have numerous anti-cancerous roles in the cell, in addition to their epigenetic modulation of oncogenetic and tumour suppressive genes (Stearns *et al.*, 2007). Understanding the complex neoplastic effects of these drugs could be beneficial to the development of improved chemotherapeutics targeted to specific cellular pathways, improving patient survival.

1.4.2 Chemical Classes of HDIs

The common mechanism by which the hydroxamic acids inhibt HDACs is through chelation of the Zn^{2+} ion at the active site; therefore, they can only affect isoforms of the class I, II and IV HDACs. It has also been demonstrated that the benzamide class access the 'foot pocket' region which is adjacent to the active site. Histone deacetylase inhibitors mimic acetylated lysine substrates and share common structural properties, including a surface recognition site necessary for contact with the active site, a linker domain that mimics acetylated substrate, and the metal binding domain, which mediates interaction with the Zn^{2+} site (Bieliauskas and Pflum, 2008; Ma *et al.*, 2009; Bressi *et al.*, 2010; Wanczyk *et al.*, 2011; Parbin *et al.*, 2013). It is the linker domain which properly positions the metal binding domain and the

capping group for required interactions with the active site of the HDAC enzyme (Bieliauskas and Pflum, 2008). It has also been shown that HDIs can block HDAC4 from entering the nucleus and thus prevent its catalytic activity within that subcellular compartment (Kong *et al*., 2011).

Based on their bidentate chelator, HDIs are grouped into four chemical classes, and include the hydroxamates, cyclic tetrapeptides, aliphatic acids and benzamides. These are either considered to be 'pan-inhibitory', isoform- and/or class-specific for HDACs (Ma *et al*., 2009; Wanczyk *et al*., 2011; Bressi *et al*., 2010; Parbin *et al*., 2013). It has been shown that cyclic tetrapeptide capping moieties and benzamide metal binding domains confer specificity to class I HDACs (Bieliauskas and Pflum, 2008), as removal of the amino group of benzamides reduced class I-specificity (Fournel *et al*., 2008). The hydroxamates are generally 'pan-specific' to class I, II and IV (Bieliauskas and Pflum, 2008).

In part due to their chemotherapeutic and chemopreventative nature, many HDIs are currently in phase I and/or phase II clinical trials (Piekarz *et al*., 2007; Lee *et al*., 2008), and two have been approved for use in the clinic. In 2006, the FDA approved Vorinostat (suberoylanilide hydroxamic acid; SAHA) for the treatment of cutaneous T-cell lymphoma, and later in 2009 the cyclic peptide Romidepsin (FK-228) had also been approved for treatment (Mann *et al*., 2007; Slingerland *et al*., 2013). In addition, Vorinostat has shown promising results for non-Hodgkin's lymphoma (Kirschbaum *et al*., 2011), and had modest effect when administered in conjunction with tamoxifen in tamoxifen-resistant breast cancers. In this phase II trial, Vorinostat treatment was able to partially reverse tamoxifen resistance (Jagannath *et al*., 2010; Munster *et al*., 2011) and re-sensitise the cells to the anti-hormonal (Sharma *et al*., 2006; Zhou *et al*., 2006; Stearns *et al*., 2007). In fact, there have been many pre-clinical phase I and/or II trials of Vorinostat in conjunction with aromatase inhibitors, anti-oestrogens or small molecule inhibitors (Fuino *et al*., 2003; Stearns *et al*., 2007; Munster *et al*., 2011; Slingerland *et al*., 2013).

It has also been suggested that dual treatment of mesenchymal cells with Vorinostat and the aminoflavone pro-drug APF464 can be clinically beneficial in breast cancer. The HDI sensitises triple-negative breast cancer cells to APF464 treatment, likely through a process involving HDI-mediated ERα re-expression. It was illustrated that Vorinostat induced ERα mRNA levels after 24 hr treatment, restoring aminoflavone responsiveness. Mouse xenograft experiments also supported these *in vitro* observations. Therefore, HDIs have the ability to alter gene expression of the triple-negative MDA-MB-231 and Hs578T cell lines, producing a less

aggressive phenotype (Stark *et al*., 2013). In addition, a pre-clinical study illustrated that co-treatment of breast cancer cells with an HDI and tamoxifen triggers apoptosis (Thomas *et al*., 2011). Furthermore, in a phase II study, the benzamide Entinostat has shown promise when administered in conjunction with the aromatase inhibitor exemestane in ERα-positive metastatic breast cancer. In September 2013, it had been labelled as a 'breakthrough therapy' by the FDA (Yardley *et al*., 2013).

As previously indicated, butyrate was the first identified HDI, followed by Trichostatin A (TSA) in the 1990s (Tsuji *et al*., 1975; Yang *et al*., 2007; Waldecker *et al*., 2008; Ma *et al*., 2009). Presently, there are many newly-developed synthetic compounds with diverse structures being tested in the laboratory that harbour HDI-activity (Yang *et al*., 2007; Ma *et al*., 2009). There has also been a focus on synthesising isoform- or class-specific HDIs, but due to the high sequence homology observed between isoforms, it has been a challenging process (Bieliauskas and Pflum, 2008). As such, while there are many different classes and types of inhibitors, only those of interest will be discussed below.

Trichostatin A (Figure 1.4 A) is an anti-fungal antibiotic isolated from *Streptomyces hygroscopious* and interacts at nanomolar concentrations directly with the Zn^{2+} ion at the active site of HDACs (Tsuji *et al*., 1975; Butler and Kozikowski, 2008). It belongs to the hydroxamates chemical class, shares structural similarity to Vorinostat and is able to inhibit class I, II and IV HDACs. Therefore, TSA is considered a 'pan-inhibitory' HDI (Haggarty *et al*., 2003; Duvic *et al*., 2007; Khan *et al*., 2008; Slingerland *et al*., 2013). In many cancer cell lines, TSA has been shown to increase histone acetylation, apoptosis and induce $p21^{WAF1}$ expression, and exhibit anti-proliferative properties (Vigushin *et al*., 2001; Glaser *et al*., 2003). Interestingly, ERα-positive breast cancer cell lines were more sensitive to the drug, indicating that TSA could target different mechanisms of growth between the ERα-positive and -negative cell lines (Vigushin *et al*., 2001). In addition, the loss of ERα expression in ERα-negative cancers can be associated with loss of methylation of the promoter (Lapidus *et al*., 1998; Stearns *et al*., 2007). TSA reactivated ERα expression and functions synergistically with the DNMT inhibitor AZA to re-express ERα mRNA. HDI treatment results in acetylated histones at the ERα promoter in MDA-MB-231 breast-derived cells. In addition, the progesterone receptor, an ERα responsive gene, was induced in these cell lines (Yang *et al*., 2001).

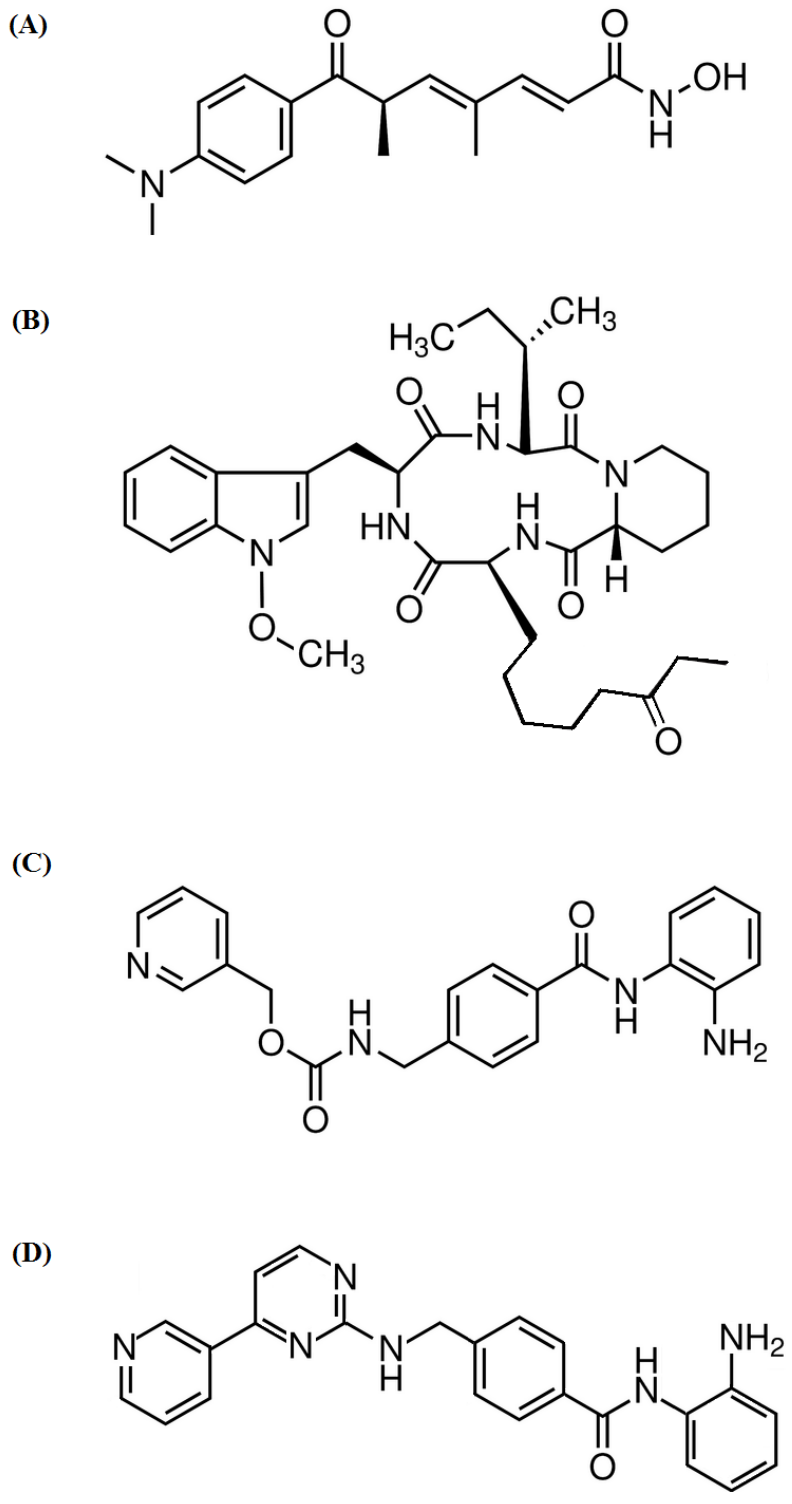

Figure 1.4. The chemical structures of histone deacetylase inhibitors. Depicted are the structures of (A) TSA, a hydroxamic acid, (B) Apicidin, a cyclic tetrapeptide, and (C) Entinostat and (D) Mocetinostat, the benzamides.

Apicidin (Figure 1.4 B) is a fungal metabolite with anti-protozoan activities isolated from *Fusarium* species (Darkin-Rattray *et al*., 1996). It is a cyclic tetrapeptide with specificity for class I HDACs, particularly HDACs 1, 2 and 3, in the nanomolar range (Furumai *et al*., 2002; Khan *et al*., 2008). However, despite its classification as a class I-specific HDI, Apicidin treatment decreased protein expression of HDAC4 in the SK-OV-3 ovarian cancer cell line. This correlated with diminished invasive properties and altered gene expression profiles involving MMP-2 (metalloproteinase-2) and RECK (reversion-inducing-cysteine-rich protein with kazal motifs) levels. It was further observed that repression of HDAC4 abrogated its binding to the Sp1 binding elements of the RECK gene promoter (Ahn *et al*., 2012). Apicidin treatment of ERα-positive breast, endometrial and ovarian cancer cell lines increases acetylation of H3 and H4, in addition to inducing cytotoxicity, apoptosis and cell cycle arrest at the G1 and/or G2/M phases. This growth inhibition was likely achieved through the down-regulation of cyclins and cyclin-dependent kinases, and the up-regulation of the G/S phase regulators, $p27^{KIP1}$, $p16^{INK}$ and $p21^{WAF1}$ (Ueda *et al*., 2006; Jy *et al*., 2008; Khan *et al*., 2008; My *et al*., 2009). It was additionally observed that these cells underwent intrinsic apoptosis through up-regulating BAX (Jy *et al*., 2008; My *et al*., 2009). This Apicidin-mediated cell death and proliferative inhibition was selective to cancer cell lines, as a non-cancerous endometrial cell line was far more resistant to cytotoxic effects (Ueda *et al*., 2006).

The two benzamides, Entinostat (MS-275) (Figure 1.4 C) and Mocetinostat (MGCD0103) (Figure 1.4 D) are specific for class I HDACs in the nanomolar to micromolar range (Fournel *et al*., 2008; Khan *et al*., 2008); however, it has been shown that they have more activity toward HDAC1 than HDAC3 (Hu *et al*., 2003; Beckers *et al*., 2007; Ropero and Esteller, 2007; Bieliauskas and Pflum, 2008), with HDAC8 only inhibited at high micromolar concentrations (Khan *et al*., 2008; Beckers *et al*., 2007). Interestingly, Mocetinostat also showed activity against the class IV HDAC11 in the high nanomolar to low micromolar range (Kalita *et al*., 2005; Fournel *et al*., 2008), while Entinostat harbours specificity toward HDAC9 (Binder and Lee, 2008). Mocetinostat and Entinostat have entered clinical trials, and as previously mentioned, the FDA has labelled Entinostat as 'breakthrough therapy' (Yardley *et al*., 2013). Furthermore, in a phase II clinical trial evaluating Mocetinostat's efficacy against relapsed classical Hodgkin's lymphoma, it was found that the chemotherapeutic was well tolerated with promising results (Younes *et al*., 2011). It also exhibited anti-tumour effects, such as cell death and an autophagic

response (Boumber *et al*., 2011).

Both Entinostat and Mocetinostat induce histone acetylation, cell cycle arrest, $p21^{WAF1}$ expression, cellular differentiation and apoptosis in a range of cancer cell lines, such as breast, colorectal, lung and leucaemia (Beckers *et al*., 2007; Fournel *et al*., 2008; Kelly and Cowley, 2013). It has also been shown that cell lines derived from normal breast tissue are resistant to cytotoxic and anti-proliferative effects of these benzamides (Fournel *et al*., 2008). In xenographs of immune-compromised mice, Mocetinostat inhibited tumour growth and reduced tumour volume (Fournel *et al*., 2008), and Entinostat has shown *in vitro* activity against trastuzumab-resistant HER2-amplified cell lines. In fact, co-treatments of Entinostat with the HER2-antibody have synergistic effects wherein Entinostat re-sensitises the cells to trastuzumab-mediated cell cycle arrest and apoptosis (Huang *et al*., 2011). In addition, Entinostat combined with the HER2/*neu* kinase inhibitor lapatinib resulted in synergistic inhibition of proliferation, colony formation and tumour size *in vivo*. In a similar manner, Entinostat re-sensitised trastuzumab- or laptinib-resistant cells to the aforementioned treatment, and enhanced growth arrest and apoptosis by FOXO3-mediated BIM1 expression (Lee *et al*., 2014). It has also been established that Entinostat can reverse the epithelial-to-mesenchymal transition in triple-negative breast cancer cell lines via up-regulation of E-cadherin. Potentially, Entinostat can reduce invasive and metastatic potential of tumours (Shah *et al*., 2014).

Particularly relevant to ERα-positive breast cancers, it has been shown that these inhibitors are able to repress ERα and PgR expression in breast cancer cell lines. Inhibition of HDAC2 leads to the down-regulation of PgR. Enhanced apoptosis in ERα-positive cell lines was observed when HDIs were combined with tamoxifen. Thus, HDIs act synergistically with anti-hormonal therapies to induce apoptosis in certan subtypes of breast cancer cell lines (Bicaku *et al*., 2008). As highlighted earlier, HDI treatment differentially affects ERα expression in hormone-receptor positive and negative cell lines; it up-regulates ERα in ERα-negative cell lines, but represses expression in ERα-positive cell lines. Furthermore, 'pan-specific' or class I-specific drugs also reduced PgR mRNA and protein expression, whereas both class I and II inhibition affected ERα expression (Duong *et al*., 2006; Hodges-Gallagher *et al*., 2006; Sharma *et al*., 2006; Zhou *et al*., 2007; Bicaku *et al*., 2008).

1.4.3 Differential Effects of HDI Treatment

It has been well-established in the literature that global histone acetylation is observed upon HDI treatment (Glaser *et al*., 2003; Beckers *et al*., 2007; Ueda *et al*., 2007; Im *et al*., 2008; Fournel *et al*., 2008; Khan *et al*., 2008; Ahn *et al*., 2009; Wang *et al*., 2009; Kelly and Cowley, 2013). However, it has recently been found that activated genes do not necessarily harbour enhanced acetylation at their promoters or within coding regions. Therefore, despite HDI-mediated up-regulation of gene expression, the associated 'active' histone acetylation marks were non-significant (Halsall *et al*., 2012). There is also evidence that HDI treatment only results in transient acetylation (Rada-Iglesias *et al*., 2007) and that this affects genes which are already in the transcriptionally active state (Wang *et al*., 2009).

It is likely that HDIs exert the aforementioned effects via binding to the multi-protein complexes that HDACs function in, and it has been observed that HDIs have varied affinities for the subunits and HDAC enzymes. These included HDIs with higher affinities for certain isoforms or complex subunits, as well as isoforms which did not respond to some HDIs. For example, the hydroxamates and cyclic tetrapeptides exhibited higher specificity for the Sin3 complex, while the benzamides had preference to the NCoR/SMRT and HDAC3 complex. It was also observed that HDIs interact with different co-repressors, although these multi-protein complexes share the same catalytic HDAC subunit (Bantscheff *et al*., 2011).

Furthermore, despite their shared ability to inhibit HDAC enzymes, there are only a small percentage of similarities between altered gene expression profiles following drug treatment. As previously highlighted, these inhibitors belong to several chemical classes based on their structures. Thus, the differential effects of HDAC inhibitors question the non-histone and non-transcriptional targets of these compounds. For instance, the transcriptional responses mediated by HDIs could be due to the acetylation of transcription factors that then act upon gene promoters. It has also been observed *in vitro* that only certain HDIs, such as TSA and Vorinostat, are pro-apoptotic, while other inhibitors had no effect. However, these HDIs could inhibit cell cycle progression at the G2/M phase (Halsall *et al*., 2012). Distinct biological effects of 'pan-inhibitory' hydroxamate HDIs have also been observed between two inflammatory breast cancer cells. In addition to differential sensitivities between the cell lines, there were altered gene signatures that resulted in apoptosis and cell cycle arrest (Halsall *et al*., 2012; Chatterjee *et al*.,

2013).

In breast and bladder cell lines, hydroxamates such as TSA and Vorinostat, were associated with different gene expression signatures than the benzamide Entinostat, and only a small subset of genes were modulated similarly by all three HDIs in multiple cell lines. These genes were most often associated with apoptosis and cell cycle inhibition (Glaser *et al*., 2003). Numerous studies have also highlighted differential HDI-mediated gene signatures between the same chemical classes, wherein less than 10% of genes were similarly altered. The treatment of two inflammatory breast cancer cell lines with hydroxamic acids, TSA and CG-1521, inhibited the cell cycle, albeit at different cellular checkpoints (G1 versus G2/M), and initiated apoptosis, but exhibited variations between gene expression. There were also diverging effects on cytoskeleton and actin acetylation, leading to cytoskeletal abnormalies. It was postulated that these differences were due to the heterogeneity of the breast cancer cell lines and complement of expressed HDACs (Chatterjee *et al*., 2013).

Studies involving selective knock-down of individual HDAC enzymes and class-specific treatment with HDIs have illustrated additional differential effects of HDIs. For example, while the short-chain fatty acid valproate and the hydroxamic acid Belinostat are both class I-specific compounds, they affected the regulation of different sets of genes in the HeLa cell line. Knock-down of HDACs 1 through 3 didn't mimic HDI-mediated gene effects within the cells, and resulted only in reduce viability with no effect on cell cycle progression (Dejligbjerg *et al*., 2008). Furthermore, in colorectal cell lines, 'pan-inhibitory' hydroxamates Vorinostat and LBH589, shared similar cellular effects such as apoptosis, histone acetylation and cell cycle arrest, but differentially responded with respect to gene expression. A small percentage of genes associated with mitosis, anti-angiogenesis and anti-apoptosis were similarly regulated between two cell lines (LeBonte *et al*., 2009).

In addition to HDIs inhibiting HDACs, it is likely that they inhibit other Zn^{2+}-dependent enzymes, and could likely exert their effects through additional pathways. It has recently been demonstrated that Vorinostat disrupts the Sin3 co-repressor complex through dissociation of the Ing2 subunit. Thus, in addition to the effects of these compounds on the inhibition of HDAC enzymes, they also possess off-target effects and disrupt the integrity of the co-repressor complexes (Sardiu *et al*., 2014). Furthermore, HDACs are able to act within the cell in the absence of their deacetylase ability. It is also interesting to note that HDAC knock-out does not

phenocopy HDI treatment (Dejligbjerg *et al*., 2008; Sun *et al*., 2013). For example, it has been shown that simultaneous knock-down of HDAC1 and Belinostat treatment negated the apoptotic effects of HDI treatment, which was not observed with knock-down of other class I HDACs (Dejligbjerg *et al*., 2008).

1.5 Histone Deacetylase Inhibitor-Mediated Transcriptional Regulation

1.5.1 $p21^{WAF1}$

In 1993, three independent research groups identified a gene whose protein induction was involved in growth suppression and associated both with wild-type p53 and cyclin-dependent kinase (CDK)/cyclin complexes. This gene encoded a 21 kDa protein and was named p21 WAF1 (wild-type associated factor-1) and p21 CIP1 (CDK-interacting protein) (el-Deiry *et al*., 1993; Harper *et al*., 1993; Lagger *et al*., 2003). It will be referred to as $p21^{WAF1}$ in this thesis. It was illustrated that this gene contained a p53 binding site located 2.4 kilobase-pairs upstream of the coding region (el-Deiry *et al*., 1993), and interacted with cyclins A, D1 and E with CDK2 in co-immuno-precipitation experiments (Harper *et al*., 1993). In addition to p53-dependent transcription, $p21^{WAF1}$ is transcribed in a non p53-dependent manner.

The cell cycle is tightly regulated through the activity of cyclin/CDK complexes and the cyclin-dependent kinase inhibitors (CKIs) (Jung *et al*., 2010; Warfel and el-Deiry, 2013). These cyclin and CDK complexes function to phosphorylate pRb and thus induce expression of E2F regulatory genes involved in cell proliferation. The CKIs are a family which includes the WAF1/CIP/KIP members, consisting of $p21^{WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$, and share conserved domains at their amino-terminal region, with variable regions at the carboxy-terminal region (Gartel and Tyner, 2002; Warfel and el-Deiry, 2013). The cyclin-dependent kinase inhibitor $p21^{WAF1}$ negatively regulates the progression of the cell cycle through inhibition of cyclin/CDK2 complex activity (Sherr and Roberts, 1999). The over-expression of $p21^{WAF1}$ is associated with cell cycle arrest at the G1, G2 or S phases (Gartel and Tyner, 2002), and dysregulation of $p21^{WAF1}$ is frequently observed in various tumours, leading to cellular growth advantages (Figure 1.5) (Warfel and el-Deiry, 2013). It has been extensively documented in the literature that HDI treatment induces $p21^{WAF1}$ expression in tumour cells (Ocker and Scheider-Stock, 2007; Gartel

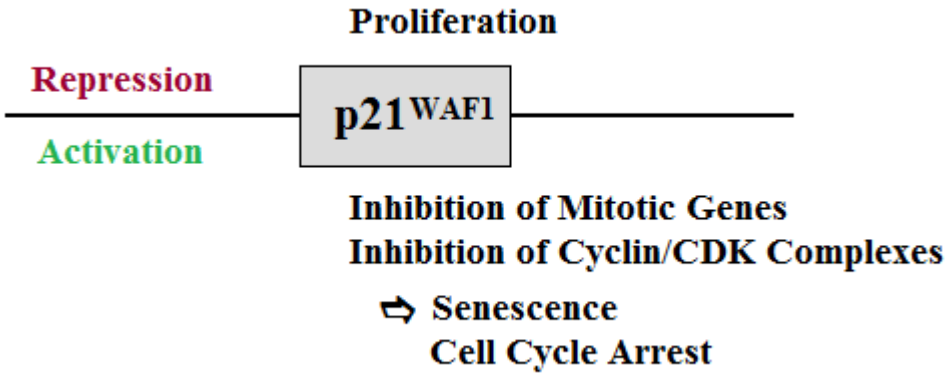

Figure 1.5. Functions of the p21^{WAF1} tumour suppressor gene. When activated, p21^{WAF1} leads to cell cycle arrest through inhibiting the action of cyclin/CDK complexes and repressing pro-mitotic genes. Alternatively, when de-regulated, the repressive qualities of p21^{WAF1} are reversed and proliferation abnormally progresses.

and Tyner, 2002).

1.5.1.1 HDI-Mediated Induction of $p21^{WAF1}$

The promoter of $p21^{WAF1}$ has six conserved GC boxes that bind the Sp1 family of transcription factors. It has further been demonstrated that a critical region -78 and -72 upstream is required for HDI-mediated activation (Sowa *et al*., 1997; Huang *et al*., 2000; Lagger *et al*., 2003). It is thought that the growth inhibition mediated by $p21^{WAF1}$ induction contributes to the anti-cancerous effects of these chemotherapeutics. While $p21^{WAF1}$ expression is activated both in a p53-independent and -dependent manner, the activation of $p21^{WAF1}$ following HDI treatment is independent of the tumour suppressor protein p53 and new protein synthesis (Lagger *et al*., 2003; Simboeck *et al*., 2010). In addition, the up-regulatory effects that these compounds have on $p21^{WAF1}$ expression are likely due to the accumulative effect of several mechanisms.

It has previously been illustrated that Vorinostat-mediated induction of $p21^{WAF1}$ is likely through the two Sp1 binding sites at the promoter, and that Vorinostat failed to alter the binding affinity of the Sp1 and Sp3 transcription factors to these sites (Huang *et al*., 2000). Furthermore, Vorinostat treatment modified the acetylation and methylation of histones at the $p21^{WAF1}$ promoter, an effect absent in genes unaffected by HDI treatment. This was accompanied by a decrease in HDAC1 and increase in RNA pol II at the promoter region (Gui *et al*., 2003). In addition, enhanced $p21^{WAF1}$ expression due to TSA administration is mediated through MAP kinase (MAPK) signalling, resulting in histone H3 serine 10 (H3S10) phosphorylation at the promoter region of $p21^{WAF1}$. This preceeded the acetylation of H3K14 and RNA pol II binding. However, acetylation mediated by TSA is not sufficient for activation; MAPK signalling and phosphorylation of H3S10 is additionally required (Simboeck *et al*., 2010). Furthermore, disruption of the Ing2 and Sin3 complex mediated by Vorinostat abrogates the co-repressor binding to the $p21^{WAF1}$ promoter, contributing to the anti-proliferative effects of these compounds (Smith *et al*., 2010).

It has also been shown that $p21^{WAF1}$ is silenced when HDAC1 and PP2A associate with co-repressor complexes and the Sp1 and Sp3 transcription factors at the promoter (Sun *et al* 2002; Simboeck *et al*., 2010). In addition, the protein kinase CK2 associates with Sp3-recruited HDAC2 to phosphorylate it and increase oestrogen-dependent proliferation in breast cancer cells

(Sun *et al*., 2002). Furthermore, HDAC1 regulates cellular proliferation and represses $p21^{WAF1}$ in embryonic mouse cells; *hdac*-null mouse embryos exhibited increased $p21^{WAF1}$ expression and hindered proliferation. In this way, HDAC1 is involved in cellular proliferation mediated through $p21^{WAF1}$, as previously highlighted (Lagger *et al*., 2003; Gui *et al*., 2004; Senese *et al*., 2007; Zupkovitz *et al*., 2010).

While $p21^{WAF1}$ is universally up-regulated with HDI treatment (Gartel and Tyner, 2002; Ocker and Scheider-Stock, 2007; Simboeck *et al*., 2010), gene repression mediated by these compounds are more diverse and dependent upon the inhibitor utilised. The Bonham Lab has demonstrated that SRC, and more recently MYC, are transcriptionally repressed in cancer-derived cell lines following treatment with 'pan-specific' HDIs, such as TSA and sodium butyrate (NaB) (Kostyniuk *et al*., 2002; Dehm and Bonham, 2004; Bonham and Beaton-Brown, unpublished data). This repression is independent to imbalances in the HAT/HDAC ratio and inhibition of HDAC enzymes, and is likely due to non-target effects that these compounds have within the cell. Both SRC and MYC are proto-oncogenes and will be discussed in more detail below.

1.5.2 SRC

The proto-oncogene SRC encodes the non-receptor tyrosine kinase $pp60^{Src}$ (Src) and is the founding member of the Src-family kinases (SFKs). It is the cellular homologue of the oncogenic viral protein v-Src, the avian Rous Sarcoma virus isolated from poultry (Rous, 1911; Brown *et al*., 1996; Wheeler *et al*., 2009), and since its discovery in 1976, several homologues have been identified in the human genome. These SFKs include the ubiquitously expressed Fyn and c-Yes, as well as the haematopoietic-specific kinases Hck, Lck, Yrk, Blk, c-Fgr and Lyn (Cance *et al*., 1994; Lee *et al*., 1994; Oberg-Welsh and Welsh, 1995; Thuveson *et al*., 1995; Parsons *et al*., 2004; Roskoski, 2004; Wheeler *et al*., 2009). Through downstream mediators, Src is involved in myriad oncogenic cellular processes, such as proliferation, cell motility, angiogenesis, cell-cell adhesion, survival and differentiation (Thomas and Brugge, 1997; Biscardi *et al*., 1999; Frame, 2004). Thus, over-expression and/or enhanced activity of Src signalling is commonly observed in many cancers. In fact, it has been illustrated both in our laboratory and other research groups that Src is up-regulated and activated in many cancer-derived cell lines and

contributes to tumorigenic processes (Cartwright *et al*., 1989, 1990; Iravani *et al*., 1998, Brunton *et al*., 1997, Dehm and Bonham, 2004; Alvarez *et al*., 2006; Wheeler *et al*., 2009). While these processes will be discussed in greater detail in a forthcoming section, it was further determined that this over-activation is resultant from enhanced transcription of the SRC gene (Dehm *et al*., 2001). This transcriptional activation of SRC and its repression by certain HDIs has become a focus in our laboratory.

1.5.2.1 The Regulation of SRC and Src

1.5.2.1.1 SRC Transcriptional Regulation

SRC contains 14 exons and is transcribed from two alternative promoters associated with a unique 5' UTR (Figure 1.6). These promoters, which were labelled 1A and 1α, are 1 kilo-basepairs away from one another. SRC 1α is tissue-specific to the stomach, liver, kidney, prostate and pancreas, while the 1A promoter is ubiquitously expressed. While both the SRC promoters have an initiator element and lack a TATA box, they are controlled by different mechanisms critical for function. The 1α promoter is regulated by the transcription factor HNF-1 (hepatocyte nuclear factor-1), whereas the housekeeping 1A promoter has a high GC content and is controlled by the Sp1 family of transcription factors through the GC1 and GA2 sites. SRC 1A also associates with the hnRNP K through three polypurine:polypyrimidine tracts (Bonham *et al*., 1993, 2000).

It has been illustrated that both promoters are active and able to control transcription in cancer-derived cell lines, although certain preferences exist. For instance, the T47D breast cancer cell line transcribes SRC predominantly from the 1A promoter while the Colo201 colorectal cells utilise both 1A and 1α (Bonham *et al*., 2000). Despite the different regulatory mechanisms of the 1A and 1α promoters, both are repressed equally by certain HDIs, such as NaB and TSA. This down-regulation did not depend on neo-protein synthesis and was observed at both the RNA and protein levels (Kostyniuk *et al*., 2002). As mentioned, this HDI-mediated repression has been extensively investigated in our laboratory.

It has also been found that SRC expression is down-regulated following HDI treatment (Kostyniuk *et al*., 2002; Dehm and Bonham, 2004), an effect which was also observed in

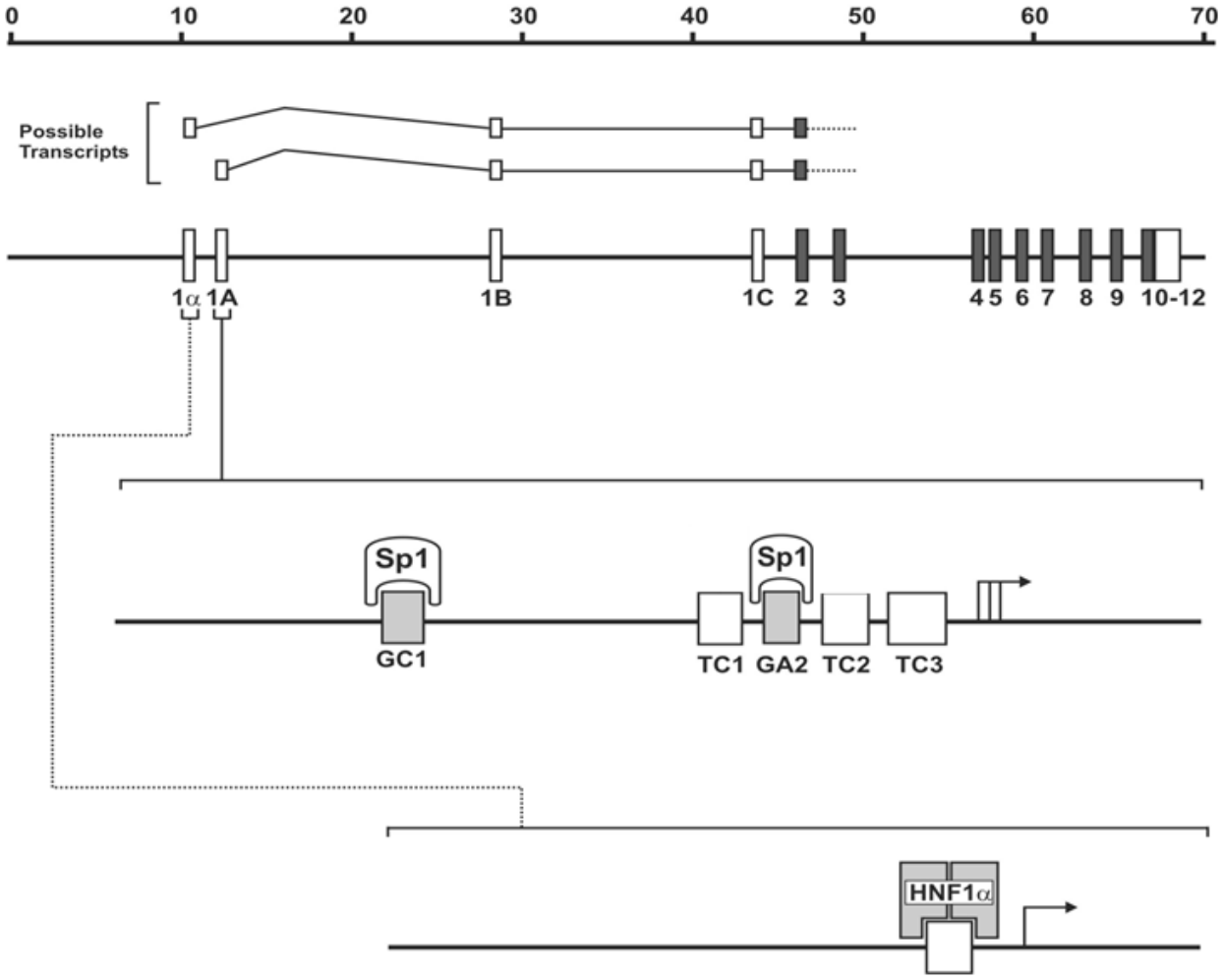

Figure 1.6. The organisation of the SRC gene. Indicated are the 1α and 1A promoters, controlled by HNF-1α and the Sp1 family of transcription factors.

additional SFK members. HDI-mediated down-regulation of gene expression occurred upon TSA and NaB treatment in a dose- and time-dependent manner in colorectal and hepatocarcinoma cell lines (Kostyniuk *et al*., 2002; Dehm and Bonham, 2004; Hirsch *et al*., 2006). This down-regulation of mRNA expression occurred through transcriptional repression independent of new-protein synthesis (Hirsch *et al*., 2006). Recent research in the Bonham Lab has illustrated that it is likely due to a post-initiation event stalling RNA pol II at the promoter. In fact, chromatin immuno-precipitation (ChIP) experiments performed by PhD candidate Erika Beaton-Brown have illustrated that H3 acetylation and H3K4 tri-methylation increases at the promoter of SRC, suggesting activation of the gene. In addition, ChIP analyses have indicated that RNA pol II is present at the promoter, whereas H3K36 tri-methylation, a marker of elongation, is decreased across the coding region of the gene. The presence of the negative elongation factor NELF is also increased at the promoter, indicating a promoter proximal paused gene. These observations have also been demonstrated for two additional genes regulated in a similar manner to SRC, in particular the oncogenic transcription factor MYC (Bonham and Beaton-Brown, unpublished data).

1.5.2.1.2 Src Regulation

The structures of the SFKs are highly conserved and diverge only in their amino-terminal 'unique' domain. Src family kinases are targeted to the plasma membrane through myristoylation of the Src homology 4 (SH4) domain, amino-terminal to the unique domain. The Src homology 3 (SH3) domain, which binds polyproline-rich sequences, and the Src homology 2 (SH2) domain are adjacent to a polyproline-rich linker domain (Cooper and King, 1986; Roskoski *et al*., 2004; Okada, 2012). The SH2 domain, highly conserved among species, recognises phospho-tyrosine proteins and contains an arginine molecule that contributes to the electrostatic interactions (Eck *et al*., 1993; Watts *et al*., 1993; Boggon and Eck, 2004), and the SH3 domain contains hydrophobic and aromatic amino acids that recognise 'PxxP' sequences (Feng *et al*., 1994; Lim *et al*., 1994; Boggon and Eck, 2004). The catalytic tyrosine kinase domain contains a positive regulatory tyrosine residue in the activation loop, whereas the carboxy-terminal tail region contains a negative regulatory tyrosine (Cooper and King, 1986; Roskoski *et al*., 2004; Okada, 2012).

Src contains three domains which are essential for proper regulation and function, and is controlled through reciprocal phosphorylation and dephosphorylation of the key regulatory tyrosine residues (Copper and King, 1986; Courtneidge, 1985; Okada and Nakagawa, 1989; Okada, 2012). In normal resting cells, Src is tightly locked into a closed conformation through interaction of phosphorylated tyrosine 530 in the carboxy-terminal tail with the SH2 domain, mediated by the negative regulator of Src function, the cellular Src kinase (Csk) (Copper and King, 1986; Courtneidge, 1985; Okada and Nakagawa, 1999). The intra-molecular interaction of the phospho-tyrosine with the SH2 domain properly positions the additional interaction of the SH3 domain with the polyproline-rich linker domain, contributing to the auto-inhibitory 'locked' state by burying the positive regulatory tyrosine 417 (Williams *et al*., 1997; Xu *et al*., 1997; Boggon and Eck, 2004). Src is activated through a series of dephosphorylation and phosphorylation events upon signalling effectors, which interact with the SH2 domain to destabilise the intra-molecular interactions and unlock the conformation of Src. This leads to the dephosphorylation of tyrosine 530 and autophosphorylation of tyrosine 417, which is no longer buried within the protein structure. Upon activation, Src can interact with and phosphorylate downsteam effectors and propagate cellular signalling, leading to neoplasticity (Courtneidge, 1985; Cooper and King, 1986; Kmiecik *et al*., 1988; Okada and Nakagawa, 1989; Wheeler *et al*., 1999; Okada, 2012).

1.5.2.2 The Role of Src in Cancer

Due to the role of Src in tumorigenic processes such as increased proliferation, cell motility, angiogenesis, cell-cell adhesion, cell survival, it is unsurprising that it is up-regulated in cancer. In fact, the over-expression and/or aberrant activity of Src has been observed in many cancers and cell lines derived from cancerous tissue, including breast and colorectal, and mediates pathways that lead to cellular proliferation and angiogenesis (Cartwright *et al*., 1989, 1990; Dehm and Bonham, 2004; Alvarez *et al*., 2006; Wheeler *et al*., 2009). Interestingly, it was shown early in the literature that NIH-3T3 murine cells transfected with either chicken SRC, Rous Sarcoma v-Src or recombinant Src plasmids constructed from the 5' of SRC and the 3' of v-Src exhibited differential transformation properties, indicating that oncogenic potential of SRC is dependent upon additional signalling. Although SRC transfected cells exhibited increased

tyrosine kinase activity and morphological characteristics differentiating them from normal untransformed cells, SRC was unable to transform the cells. However, v-Src and recombinant-Src exhibited transformative properties (Shalloway *et al*., 1984). It was then speculated that the oncogenic potential for v-Src lies within the 3' region, which differs from Src in that it lacks the carboxy terminal tail and therefore the negative regulatory tyrosine (Shalloway *et al*., 1984; Cooper *et al*., 1986; Courtneidge *et al*., 1987; Okada *et al*., 1999).

It has been established for approximately two decades that the kinase activity of Src is significantly increased in carcinoma compared to normal tissue (Cartwright *et al*., 1990; Ottenhoff-Kalff *et al*., 1992; Talamonti *et al*., 1993). Furthermore, Src activation is an early event in carcinogenesis (Cartwright *et al*., 1990) and can lead to metastasis (Talamonti *et al*., 1993). It has been observed that this enhanced activity is mediated by enhanced transcription of the gene and/or the autophosphorylation of the activating tyrosine 416 site (Kmieck *et al*., 1988; Dehm and Bonham, 2004). Through phosphorylating downstream signal effectors, Src contributes to the progression of a variety of oncogenic signalling processes, such as cellular proliferation, angiogenesis, invasion and migration (Figure 1.7) (Frame, 2004; Li *et al*., 2009).

While up-regulation of Src alone cannot transform cells or prolong tumour growth in mice (Shalloway *et al*., 1984; Luttrell *et al*., 1988; Biscardi *et al*., 2000), Src is thought to contribute to oncogenic processes by activating its downstream signalling pathways (Guy *et al*., 1994; Biscardi *et al*., 2000). Src can interact with various receptors and signalling molecules, and is frequently up-regulated with other oncogenic proteins (Maa *et al*., 1995; Tice *et al*., 1999). For instance, Src is required for the polyomavirus middle T oncogene to initiate mammary tumour development in transgenic mice (Guy *et al*., 1994). It has also been well-established that SRC can co-operatively act with receptor tyrosine kinases, such as the human epidermal growth factor receptor 1 (EGFR) to induce tumour formation in immune-compromised mice (Maa *et al*., 1995). Both Src and EGFR have been shown to be elevated in cancer and form heterocomplexes, synergistically acting to promote cellular growth. Src potentiates EGFR-induced DNA synthesis, proliferation and tumour formation in immune-compromised mice (Maa *et al*., 1995; Tice *et al*., 1999).

In addition, there is a regulatory network between Src and ERα signalling, where oestrogen administration activates downstream pro-mitotic effectors in a manner dependent on Src kinase activity (Castoria *et al*., 1999; Biscardi *et al*., 2000). In addition to increased

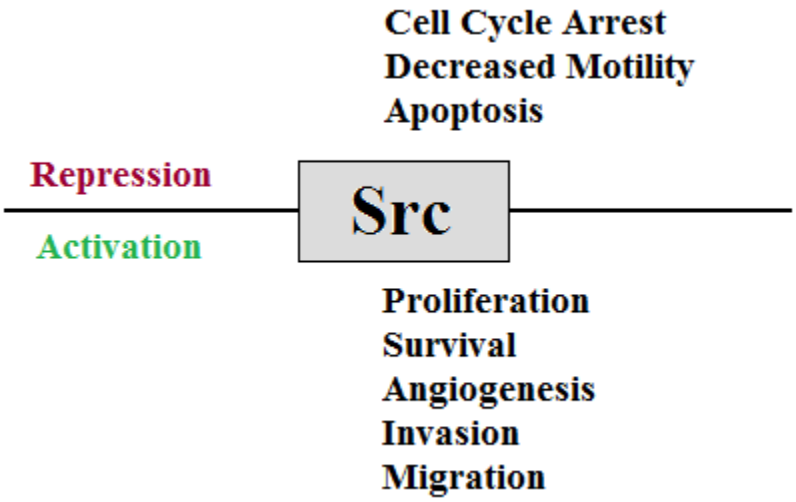

Figure 1.7. Tumourigenic and anti-neoplastic effects of Src signalling. Src is over-expressed in many cancers and leads to tumourigenic processes such as proliferation, survival and angiogenesis. The inhibition of Src through down-regulation or inactivation results in anti-cancerous effects.

proliferation, Src expression leads to the loss of intercellular adhesions (Avizienyte *et al*., 2002; Frame, 2004) as well as increased resistance to anoikis, likely through activation of the PI3K/Akt pathway. For example, colorectal cancer cell lines transfected with antisense SRC exhibited detachment-induced cellular death (Windham *et al*., 2002; Frame, 2004).

Src over-expression and increased activity can also propagate tumorigenic processes. For instance, Src-mediated phosphorylation of the Crk-associated substrate ($p130^{Cas}$ or Cas), a component of the focal adhesion complex, enhances migration and metastasis in transformed cells (Goldberg *et al*., 2003). It has been shown that phosphorylation of Cas at the YxxP motifs within its substrate domain mediates binding to SH2-domain containing effectors (Shin *et al*., 2004; Li *et al*., 2009). This process has been shown to be augmented by microRNA (miRNA), wherein active Src leads to induction of miR-224, further enhancing cellular growth. These miRNA are small, non-coding transcripts controlling gene expression and will be discussed in further detail in an upcoming section.

Interestingly, Src signalling represses miR-126, which targets the 3' UTR of Crk, another focal adhesion complex component, to inhibit Src-mediated migration of transformed cells. Furthermore, contact normalisation, a process where SRC-transformed cells regain their normal morphology upon contact with non-transformed cells, is mediated by miR-126. Therefore, Src activates Cas/Crk signalling in a dual manner, by activating Cas through phosphorylation and by decreasing miR-126 production, leading to an increase of Crk (Li *et al*., 2009).

It has been reported that enhanced Src signalling promotes latent bone metastases in primary breast cancer, and is an indicator of poor prognosis in patients with bone metastasis (Zhang *et al*., 2009, 2012). A gene expression profile associated with Src signalling correlated with increased bone metastases, independent of ERα expression, molecular subtype, differentiation and tumour grade of the primary tumour. Furthermore, as illustrated in lung- and brain-derived cell lines, the increased levels of activated Src were insignificant compared to those observed in bone metastatic-derived cell lines. Therefore, signalling from the SRC kinase, but not SFK members c-Yes and Fyn, promotes disseminated cancer cells to proliferate in the bone marrow tumour microenvironment. It is thought that Src functions through the chemokine CXCL12 to advance cell growth and lessen pro-apoptotic signalling (Zhang *et al*., 2009).

Src signalling has also been implicated in invasive properties in hormone- and growth factor-resistant ERα-positive tumours. As previously highlighted, hormone receptor-positive

breast cancers treated with endocrine therapies can develop resistance to treatment, ultimately leading to relapse and increased mortality among survivors. It has been shown that increased Src signalling is associated with endocrine therapy resistance, due to the role Src plays in cell-cell and cell-matrix functions, promoting an aggressive, invasive phenotype (Hiscox *et al*., 2006). Furthermore, Src kinase activity is amplified in metastatic, invasive cell lines (Mao *et al*., 1997; Jackson *et al*., 200l; Slack *et al*., 2001; Irby and Yeatman, 2002; Hiscox *et al*., 2006) and Src inhibition mediated by AZD0530, a Src/Abl small molecule inhibitor, negates invasion and migration in resistant cell lines. In addition, acquired resistance to tamoxifen involves Src kinase activity (Hiscox *et al*., 2006).

Due to its role and enhanced activation in cancers, Src is an attractive target in chemotherapy treatments (Dehm and Bonham, 2004; Alvarez *et al*., 2006; Wheeler *et al*., 2009). There are various small molecule tyrosine kinase inhibitors in clinical trials that have shown efficacy against treating numerous malignancies, including breast, colorectal and prostate cancers. For instance, dasatinib targets the SFKs, as well as c-Kit and platelet-derived growth factor receptor (PDGFR), to inhibit proliferation and migration *in vitro* and decrease metastatic potential and tumour growth *in vivo* (Montero *et al*., 2011). It can also lead to apoptosis, a process mediated through inactivation of Src (Vandyke *et al*., 2009). In fact, the inactivation of Src activity, mediated by inhibitors such as dasatinib, is thought to be partially responsible for the anti-cancerous effects of the small molecule tyrosine inhibitors (Ishizawar *et al*., 2004; Park *et al*., 2008).

1.5.3 MYC

The MYC proto-oncogene, discovered over three decades ago, is the mammalian homologue of the avian myelocytomayosis viral v-MYC oncogene (Bishop, 1982; Escot *et al*., 1986; Lao and Dickson, 2000; Singhi *et al*., 2012). It is located at chromosomal location 8q24.1 and transcribes three separate proteins from separate initiation sites; the major product is a 62 kDa protein (termed ‘MYC2’) and is the form frequently referred to as ‘MYC’ in the literature. MYC1 and MYC2, respectively transcribed from a non-AUG and an AUG start codon, encode proteins approximately 62-64 kDa (Henriksson and Luscher, 1996; Facchini and Penn, 1998; Xiao *et al*., 1998; Lao and Dickson, 2000). The promoter(s) of MYC shares similarity to SRC

and contains several upstream positive and negative *cis*- and *trans*-elements, such as CCCTCCCCA (CT)-elements that can bind a myriad of regulatory factors, including Sp1 and hnRNPK. Furthermore, the promoter can be acted upon by various transcription factors (Levens, 2008).

Myc is a nuclear phospho-protein transcription factor thought to co-operate with other transcription factors to control approximately 10-15% of the human genome, including those genes implicated in cell growth and transformation. While Myc regulation is tightly controlled by characteristically short protein and mRNA half-lives in non-mitotic or differentiated cells, it is up-regulated following mitogenic signals. Hence, it is often associated with increased proliferation (Spencer and Groudine, 1991; Nass and Dickson, 1997; Levens, 2008). The cellular processes controlled by Myc are diverse and include pathways such as apoptosis, differentiation, growth, adhesion, metastasis and metabolism (Figure 1.8). Although, it has been shown that those genes activated or repressed by Myc can be cell- or context-specific, and therefore Myc can therefore regulate both oncogenic and tumour suppressive functions (Nass and Dickson, 1997; Jamerson *et al*., 2004; Chen and Olopade, 2008).

Myc contains an amino-terminal transactivation domain (TAD) with two Myc homology boxes (MI and MBII) conserved across Myc family members. The MBI domain is necessary for the transactivational functions of Myc, while the MBII domain is required for transcriptional repression (Cole and McMahon, 1999; Sakamuro and Prendergast, 1999; Lao and Dickson, 2000). Additionally, it has been shown that the TAD modulates apoptosis (Chang *et al*., 2000; Lao and Dickson, 2000). The carboxy-terminus harbours a basic region (BR) and a helix-loop-helix/leucine zipper (HLH/LZ) domain, which are necessary DNA binding and hetero-dimerisation with the Max and/or TFII-I transcription factors. The transcriptional activation of Myc is mediated through the Max/Myc complexes upon binding to E-box elements in DNA sequences, whereas transcriptional repression is mediated through TFII-I/Myc complexes. These E-box elements contain central CAC(G/A)TG sequences (Roy *et al*., 1993; Nass and Dickson, 1997; Facchini and Penn; 1998; Dang 1999; Lao and Dickson, 2000).

1.5.3.1 The Role of Myc in Breast Cancer

Since its discovery, the MYC proto-oncogene has been shown to be amplified, rearranged and/or over-expressed in approximately 25% of breast cancers and is associated with tumour

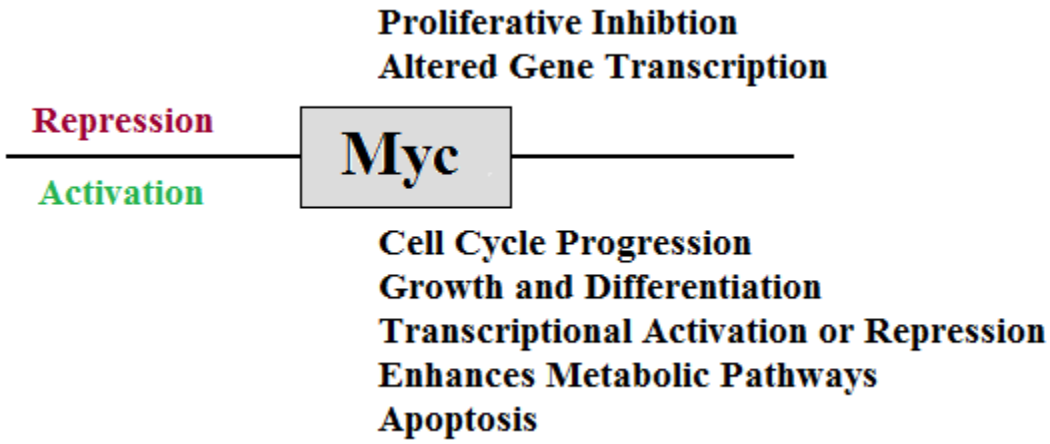

Figure 1.8. The tumourigenic functions of Myc. Myc, a transcription factor activated in numerous cancers, leads to cellular processes associated with aberrant growth mainly through gene activation and/or repression. Inhibiting Myc has been considered a viable therapeutic option due to altered gene regulation and cell cycle arrest.

aggressiveness, poor prognosis and clinical outcome (Escot *et al.*, 1986; Wong *et al.*, 1986; Dubik *et al.*, 1987; Blanchard *et al.*, 1988; Bonilla *et al.*, 1988; Garcia *et al.*, 1989; Chen and Olopade, 2008; Horiuchi *et al.*, 2012). MYC amplification is also observed in non-mammary tumours, such as colon and small-cell lung carcinoma cell lines (Little *et al.*, 1983; Escot *et al.*, 1986; Jenkins *et al.*, 1997; Visscher *et al.*, 1997). Through chromosomal analysis, it has been demonstrated that the MYC locus can be copied between 2-20 times, and is often correlated with hormone receptor-negative and basal breast cancers (Garcia *et al.*, 1989; Aulmann *et al.*, 2006). In addition, MYC-amplified tumours relapse earlier than non- amplified, and those tumours which did not exhibit MYC copy number variations showed increased copies upon relapse and metastasis (Aulmann *et al.*, 2006; Singhi *et al.*, 2012). This potentially indicates that MYC amplification is an acquired event in metastasis and that up-regulation is important for cancer progression (Singhi *et al.*, 2012).

Triple-negative breast cancers have both elevated Myc expression (Lao and Dickson, 2000) and high expression of Myc target genes over those cancers which are hormone-positive, and this correlates with a higher rate of proliferation *in vitro* and *in vivo* (Horiuchi *et al.*, 2012). Repressing Myc and its target genes in triple-negative breast cancers could be therapeutically beneficial and offers a new treatment strategies for those cancers (Chen and Olopade, 2008; Horiuchi *et al.*, 2012).

Furthermore, MYC amplification is positively associated with HER2-enrichment in breast tumour biopsies (Gaffey *et al.*, 1993; Bolufer *et al.*, 1994; Lao and Dickson, 2000), and could be implicated in tumorigenesis of ERα-positive breast cancers. The MYC promoter contains a partial ERE site and CG-rich Sp1 binding site, and upon ERα binding, induces transcription (Dubik and Shiu 1992; Nass and Dickson, 1997). In addition, oestradiol increases MYC mRNA levels in ERα-positive cell lines, a phenomenon inhibited by tamoxifen treatment (Dubik *et al.*, 1987; Wong *et al.*, 1991; Shui *et al.*, 1993; Tsai *et al.*, 1997; Lao and Dickson, 2000). Interestingly, it has also been illustrated that MYC amplification confers resistance to anti-oestrogen treatments (Venditti *et al.*, 2002) and that ERα-negative breast cancers exhibit Myc induction, which is often associated with enhanced growth (Sorlie *et al.*, 2001; Naidu *et al.*, 2002; Alles *et al.*, 2009).

Myc is moderately and spontaneously tumorigenic in mouse models; furthermore, this tumorigenicity is enhanced when another proto-oncogene is simultaneously over-expressed (Nass

and Dickson, 1997; Lao and Dickson, 2000; Rose-Hellekant and Sandgren, 2000). Transgenic mice expressing MYC under the control of the mouse mammary tumour virus (MMTV) promoter exhibit low latency mammary carcinoma development within one year of age, regardless of pregnancy or virgin status of the female mouse (Nass and Dickson, 1997; Lao and Dickson, 2000; Rose-Hellekant and Sandgren, 2000). However, co-transfection with a plasmid expressing a secondary oncogene, such as HER2/*neu*, increased both breast tumour development and the frequency of tumour formation (Stewart *et al*., 1984; Nass and Dickson, 1997; Lao and Dickson, 2000; Jamerson *et al*., 2004). In fact, tumours developed in all test sample mice, and the observed latency period reduced from one-plus year to approximately three months (Muller *et al*., 1988; Jamerson *et al*., 2004). It is interesting to note that the HER2/*neu* and MYC transgenic mice also exhibited elevated Src activity, likely due to HER2-mediated SRC activation (Cardiff *et al*., 1991; Muthuswamy *et al*., 1994; Nass and Dickson, 1997; Singhi *et al*., 2012). In addition, the tumour suppressive gene BRCA1 is able to block the transactivation properties of Myc, and BRCA1-null mice models exhibit dysregulated MYC expression, further contributing to neoplasia (Jamerson *et al*., 2004).

Additionally, MYC transgenic mouse models with homozygous deletions of the p53 tumour suppressive gene exhibit chromosomal instability and aggressive lymphoma (McCormack *et al*., 1998). Contrary, $p21^{WAF1-/-}$ null mice expressing MYC under the MMTV promoter had increased breast tumour latency and decreased incidence of tumour formation. It was postulated that $p21^{WAF1}$ acts as a modifier for Myc-mediated tumorigenesis by enhancing cyclin D expression and progression through the cell cycle. This potentially highlights the complicated role Myc plays in cancer progression (Bearss *et al*., 2002; Jamerson *et al*., 2004).

While MYC has been demonstrated to regulate numerous processes, extensive research has also indicated a role for Myc in cell cycle control and apoptosis, particularly in carcinoma cells (Nass and Dickson, 1997; Lao and Dickson, 2000; Jamerson *et al*., 2004). The over-expression of Myc is also correlated with these cellular functions (Evan *et al*., 1993; Singhu *et al*., 2012). Myc is induced in breast cancer cell lines following activation of growth factor and hormone pathways, such as through the EGF-, oestrogen- and TGFα-induced pathways; this is associated with increased proliferation (Dubik *et al*., 1987; Leygue *et al*., 1995; Nass and Dickson, 1997). While Myc is not thought to control cell cycle regulatory genes directly, as they lack the E-box motif, it is able to modulate regulation of cyclins and CDK kinase activities.

Myc-mediated cyclin E induction and $p27^{KIP1}$ repression leads to increased activity of CDK2 and reduction of phosphorylated pRb (Nass and Dickson, 1997). The inactivation of Myc reduces invasion *in vitro* and impairs metastasis *in vivo* (Wolfer *et al*., 2010).

An additional role for Myc in oncogenic processes has been shown through its involvement in apoptotic control. Myc over-expression induces apoptosis in the absence of growth-factor signalling. In the presence of growth factor signalling and Myc over-expression, apoptosis is inhibited. Myc can also activate p53 and BAX, thus preventing the survival of cells with oncogenic backgrounds (Reisman *et al*., 1993; Miyashita *et al*., 1995; Nass and Dickson, 1997). This anti-apoptotic event can be bypassed by Bcl-2-mediated pro-apoptotic signals in the presence of MYC amplification (Strasser *et al*., 1990; Bissonnette *et al*., 1992; Fanidi *et al*., 1992; Wagner *et al*., 1993; Nass and Dickson, 1997).

1.5.4 microRNA

In 1993, researchers discovered a gene encoding a pair of short RNAs in *Caenorhabditis elegans* with anti-sense complementary to the 3' untranslated region (UTR) of the developmental mRNA lin-14. It negatively regulated lin-14 through binding to the 3' UTR and inhibiting translation (Lee *et al*., 1993; Wightman *et al*. 1993; Bartel *et al*., 2004). Due to its role in *C. elegans* developmental timing, this RNA strand was termed 'short temporal RNA' (stRNA). For seven years, it appeared that lin-4 and its regulatory mechanism were particular to *C. elegans*, but the subsequent discovery of let-7 led to the identification of homologues in other species' genomes through cloning efforts. These also exhibited evolutionary conservation between the plant and animal kingdoms (Pasquininell *et al*., 2000; Brennecke *et al*., 2005; Schanen and Li, 2011).

These short RNA molecules were found to be involved in processes other than developmental timing, and therefore were then termed as 'microRNAs' (miRNAs). miRNA are small non-coding transcripts that mediate post-transcriptional regulation of target messenger RNAs (mRNAs) through semi-perfect- or perfect-complementary binding to the 3' UTR, leading to translational inhibition or mRNA decay (Bartel *et al*., 2004; Bagga *et al*., 2005). It has been found that miRNA genes constitute approximately 1% of the genome (Brennecke *et al*., 2005) and regulate one-third of protein-coding genes in biological processes, including developmental

timing, haematopoiesis, cell differentiation, apoptosis and proliferation. In addition, due to their promiscuous nature, multiple miRNA potentially target the 3' UTR of one mRNA molecule (Bartel 2004; Schanen and Li, 2011).

1.5.4.1 Transcriptional Regulation of microRNA

The genomic location of miRNA can be categorised into 'intragenic' or 'intergenic'. Simply, intergenic miRNA are located between genes, whereas intragenic miRNA are within genes. Intragenic miRNA can be found within any genomic region of the chromosome, such as within introns of protein-coding genes ('intronic'), or within introns and/or exons ('exonic') of non-coding genes (Corcoran *et al*., 2009; Ozsolak *et al*., 2009; Schanen and Li, 2011). Interestingly, due to variations in splicing, some intragenic miRNA span an exonic and intronic region (Rodriguez *et al*., 2004), and generally have different mechanisms of biogenesis than intergenic miRNA.

Intragenic miRNA commonly share their transcriptional start sites (TSS) with their host gene (Rodriguez *et al*., 2004; Ozsolak *et al*., 2009); however, it has also been shown that some intronic miRNAs are controlled by their own promoters, which are embedded within an exon of their respective host genes. Traditional mapping of potential TSS relying on RNA analyses are challenging due to the transient nature of primary miRNAs (pri-miRNAs) and their low concentration within cells. Elucidations of promoter regions of pri-miRNAs include computational approaches involving genome-wide RNA pol II binding patterns (Wang *et al.,* 2010) and ChIP-chip methods targeted for RNA pol II (Corcoran *et al.,* 2009), or active chromatin markers H3K4me and/or H3K9/14Ac (Ozsolak *et al*., 2009).

Potential promoter regions located upstream of the primary miRNA sequences for both intragenic and intergenic miRNAs were established through these assorted methods. Similarities between miRNA and RNA pol II-controlled protein-coding gene promoters were identified, such as the presence of a TATA box, GC-rich regions, TFIIB recognition elements, chromatin modifications (H3K4me and/or H3K9/14Ac) or evolutionary conservation (Ozsolak *et al.,* 2007, 2009; Corcoran *et al.,* 2009; Wang *et al.,* 2010). In addition, miRNA expression can be controlled by transcription factors; the oncogenic miR-17~92 polycistronic cluster, which is over-expressed in B-cell lymphoma, can be transcribed upon MYC binding (Mu *et al*., 2009). However, there are some miRNA promoters that have no similar sequences to the protein-coding

genes, and lack a TATA box, TFIIB recognition element or other promoter-specific chromatin markers, such as the promoter identified for the multi-cistronic cluster of miR-23a~27a~24-2 (Lee *et al.*, 2004).

Intragenic miRNA can be transcribed dependently or independently of their host gene, and regulatory sites possessing RNA pol II elements, such as CpG islands and transcription factor binding sites, have been identified upstream. A small percentage have RNA pol III regulatory sequences as well as their host TSS, and can be transcribed both by RNA pol II and III (Monteys *et al*., 2010; Corcoran *et al*., 2009). However, the majority of miRNA are intergenic and possess their own TSS (Rodriguez *et al*., 2004; Corcoran *et al*., 2009; Ozsolak *et al*., 2009; Schanen and Li, 2011).

microRNA biogenesis is a three-step process occurring co-transcriptionally and within both the nucleus and cytoplasm (Figure 1.9). Furthermore, the processes can be regulated at any stage. microRNAs are transcribed in the nucleus as long nucleotide transcripts referred to as pri-miRNA, most frequently by RNA pol II (Lee *et al*., 2004), although some miRNA undergo self-directed transcription (Song *et al*., 2010) or as previously mentioned, transcription by the short non-coding RNA pol III (Borchert *et al*., 2006). Generally, RNA pol II transcribes intragenic miRNA, whereas intergenic genes can be transcribed by both RNA pol II and III, or non-canonical transcription (Schanen and Li, 2011). Alu-elements can act as a promoter for miRNA and initiate transcription by RNA pol III (Gu *et al*., 2009), and clustered or 'polycistronic' miRNA are most often transcribed by the same TSS into one pri-miRNA molecule (Lee *et al.*, 2004). Additionally, a small fraction of miRNA can self-transcribe in the absence of a promoter or control their own expression in a negative feedback loop. For instance, mature miR-145 can activate p53, which in turn stimulates transcription of pri-miR-145 (Schanen and Li, 2011). Biogenic processing of miRNA, described in more detail below, depends on the method of transcription as well as the position of the miRNA within the genome. Additionally, miRNA expression can be regulated at both the transcription and processing levels (Lee *et al*., 2003; Yi *et al*., 2003; Zeng *et al*., 2003; Rodriguez *et al*., 2004; Lund *et al*., 2004; Schanen and Li, 2011).

Biogenesis includes cleavage of the pri-miRNA structure by the nuclear RNase endonuclease III Drosha, resulting in an approximately 65 to 70 nucleotide (nt) hairpin precursor miRNA (pre-miRNA) stem loop. At this biogenic stage, those clustered pri-miRNAs transcribed as a polycistronic unit are separated into individual pre-miRNA (Yi *et al*., 2002; Lee *et al*., 2002,

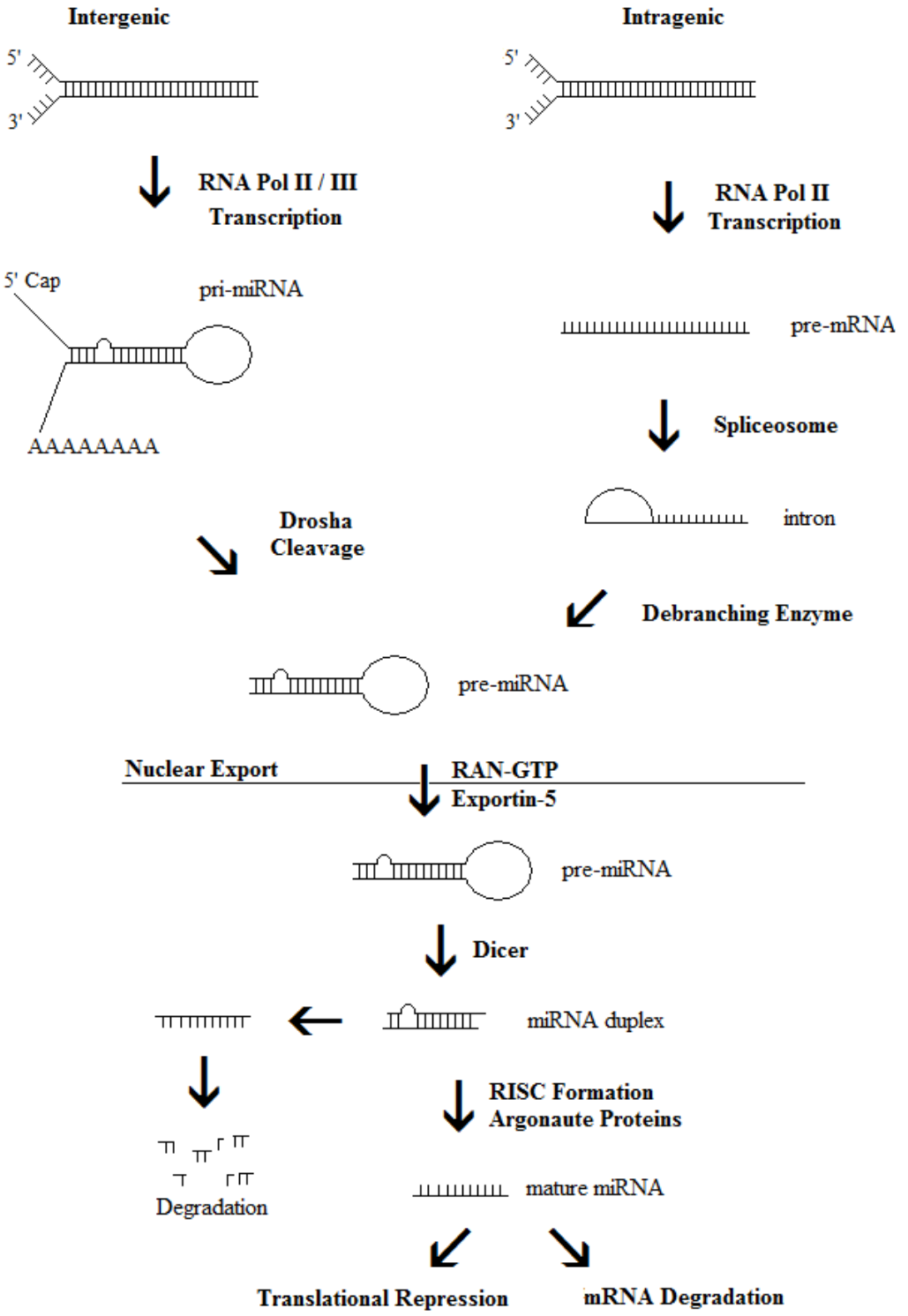

Figure 1.9. miRNA transcription and biogenesis. Schematic representation of intragenic or intergenic miRNA transcription mediated by RNA pol II and/or III and subsequent processing by Drosha and Dicer. The mature miRNA sequence is associated into the RISC complex with argonaute proteins and mediates mRNA gene expression by translational inhibition or transcript degradation.

2003; Zeng *et al*., 2002, 2003; Yi *et al*., 2003; Lund *et al*., 2004; Rodriguez *et al*., 2004; Schanen *et al*., 2011). Processing by Drosha on intronic miRNA occurs co-transcriptionally prior to splicing; both RNA pol II and Drosha have been demonstated to associate with the miRNA sequence of the gene (Morlando *et al*., 2008). In addition, RNA pol II-transcribed pri-miRNAs have a 5' cap structure and a 3' poly(A) tail, similar to protein-coding genes, which are cleaved during subsequent processing (Cai *et al*., 2004).

The pre-miRNAs are then transported to the cytoplasm through a RanGTP-dependent process utilising Exportin-5, where they are further processed into double stranded 20-25 nt duplex RNA by the cytoplasmic RNase III endonuclease, Dicer. The mature miRNA specificity comes from Dicer rather than Drosha (Lee *et al*, 2003; Yi *et al*., 2003; Zeng *et al*., 2003; Lund *et al*., 2004; Rodriguez *et al*., 2004; Schanen and Li, 2011), and further studies have illustrated that Dicer cleavage is rate-limiting and could occur several hours following pri-miRNA transcription. It is also interesting to note that in studies examining pri-miRNA transcription and mature miRNA expression, fold-changes of pri-miRNA were generally higher, thus indicating a potential post-transcriptional regulation (Castellano *et al*., 2009). Dicer recognises a double-stranded RNA molecule and cleaves two helical turns away from the terminal stem loop, leaving a double-stranded RNA with a 5' phosphate and a two nt 3' overhang. Multi-protein complexes composed of argonaute (Ago) proteins associate with the strand possessing the more stable 5' end, and this RNA-induced silencing complex (RISC) then binds to the 3' UTR of protein-coding mRNA. The seed sequence at the 5' region of the miRNA is responsible for recruiting Ago proteins (Lee *et al*, 2003; Yi *et al*., 2003; Zeng *et al*., 2003; Lund *et al*., 2004; Rodriguez *et al*., 2004; Schanen and Li, 2011). Furthermore, co-immuno-precipitation (co-IP) experiments have illustrated that Ago proteins associate with the RISC machinery and co-IP with mRNA targets; this has additionally been utilised to identify potential miRNA target genes (Karginov *et al*., 2007). The less-common strand is typically degraded, although in certain circumstances, both strands serve as functional and mature miRNA sequences; while the less-common strand had been historically annotated by an asterisk (*) after the miRNA name, it is now commonly denoted by the '-3p' strand, and the common miRNA by the '-5p' strand (Lee *et al*, 2003; Yi *et al*., 2003; Zeng *et al*., 2003; Lund *et al*., 2004; Schanen and Li, 2011). Interestingly, miRNA possessing Alu-elements frequently bypass the processing machinery, and are less-responsive to exert transcriptional regulation on their target mRNA genes. This is likely due to altered secondary structure or

adenosine to inosine editing (Hoffman *et al*., 2013).

There are several differences that occur between intronic and exonic miRNA processing. It has been shown that unspliced transcripts with exonic miRNA sequences are located in the nucleus and available for Drosha cleavage, whereas spliced transcripts are found in the nucleus and cytoplasm. Therefore, both spliced and unspliced transcripts result in pre-miRNA hairpins (Slezak-Prochazka *et al*., 2013). It is also interesting to note that pri-miRNA embedded within their host transcript can function both as a pri-miRNA and mRNA. This offers an additional level of regulation, as those pre-miRNA in the cytoplasm cannot be processed by Dicer. The miRNA stem-loop located in a 3' UTR of its host gene has the ability to moderately repress expression of the protein by sequestering it inside the nucleus, and following excision of the pre-miRNA, splicing of the introns and subsequent translation can occur. Contrary to intronic miRNA, exonic miRNA transcripts can only function as an mRNA or as a pre-miRNA, as excision of the pre-miRNA would disrupt the coding sequence (Cai *et al*., 2004). Intronic processing often occurs co-transcriptionally and in co-operation with the splicing complex producing an mRNA transcript (Cai *et al*., 2004; Kim and Kim 2007), although pri-mRNA cleavage by Drosha can also occur post-splicing in the absence of splicing machinery, and does not affect mature miRNA or mRNA production (Kim and Kim 2007). Furthermore, non-coding transcripts in the cytoplasm can serve as competing endogenous targets and sequester miRNA from their protein-coding gene targets (Cai *et al*., 2004).

Interestingly, it has recently been shown that Ago1, which is usually cytoplasmic, associates with RNA pol II and active chromatin marker H3K4me^3 at miRNA-putative genomic regions. While Ago1 presence could be found at various genetic regions, such as promoters, 5' and/or 3' UTRs, introns or exons, it was enriched at the TSS of transcriptionally active genes, particularly those involved with oncogenic processes. Due to Ago1 lacking a proper DNA-binding motif, it is speculated that miRNA are required for the interactions to occur. This is further supported by experimental knock-down of Dicer and Drosha, which reduced RNA pol II-Ago 1 binding to the chromosome. This potentially illustrates an additional regulatory role of miRNA in gene expression independent of 3' UTR binding (Huang *et al*., 2013).

1.5.4.2 microRNA-Mediated Regulation of Gene Expression

Bioinformatics have been used to predict many mRNA targets with 'miRNA response elements' (MREs). microRNA can perfectly or imperfectly bind to the 3' UTR of target protein-coding genes and lead either to mRNA cleavage and degradation, or translational inhibition, respectively (Bartel 2004; Bagga *et al*., 2005; Schanen and Li, 2011). However, it has been shown that let-7 can bind imperfectly to lin-14 and result in transcript degradation (Bagga *et al*., 2005). It is thought that the specificity of miRNA binding to the 3' UTR comes in part from the 2-8 nt region at the 5' end, referred to as the 'seed region', although other requirements and factors for target binding exist (Brennecke *et al*., 2005; Grimson *et al*., 2007). It has been shown that many miRNA recognition sites can be hindered by several factors, including the presence of AU rich base-pairs near the seed region, the adjacent proximity of co-repressive miRNA sites and/or a close distance to the stop codon. microRNA tend to act synergistically as well, and often two or more miRNA are required for optimal repression (Grimson *et al*., 2007).

mRNA:miRNA recognition sites can be classified into three categories; canonical 5' dominant sites, seed 5' dominate sites and 3' compensatory sites. Canonical and seed 5' dominant sites exhibit strong 5' nucleotide base-pairing, with either strong or weak 3' pairing, respectively, and 3' compensatory sites possess weak 5' pairing and strong 3' pairing. These classifications are evolutionary conserved, and miRNA families are typically different only in their 3' regions (Brennecke *et al*., 2005).

As mentioned previously, miRNA mediate their effects by destabilising the mRNA transcript or by inhibiting translation. mRNA degradation is mediated by decapping and depolyadenylation followed by cleavage dependent upon 5'-3' or 3'-5' exonuclease activity, respectively (Bagga *et al*., 2005), whereas translational inhibition is thought to be mediated through cytoplasmic foci known as 'p-bodies'. These are sites which contain non-translated mRNA meant for degradation. Upon RISC and miRNA binding to the 3' UTR, the miRNA are targeted for translational repression through sequestration in p-bodies. It has been shown that the Ago protein components of the RISC machinery as localised to p-bodies upon miRNA binding (Liu *et al*., 2005; Sen and Blau, 2005).

Additionally, *in vitro* transfection experiments have shown that miRNA can influence gene expression in a way that is reminiscent of transcriptional activators. For instance, following

transfection of duplex miR-373, it binds to the 5' UTR of complementary sequences found in the E-cadherin and CSDC2 promoters. This increases RNA pol II presence along the promoter region and enhances gene expression. However, this phenomena was cell-line specific (Place *et al*., 2007).

1.5.4.3 Role of Tumour Suppressive and Oncogenic microRNA in Tumorigenesis

In the last decade, there has been extensive research into the role of miRNAs in cancer, and many researchers have reported oncogenic and tumour suppressive miRNA can influence processes such as proliferation, invasion, metastasis and apoptosis (Iorio *et al*., 2005; Lu *et al.*, 2005; Scott *et al.*, 2006; Iorio *et al.*, 2007; Shenouda and Alahari, 2009; Pang *et al*., 2010; Shih *et al*., 2011). It is suggested miRNAs could play a role in the pathogenesis of various cancers and therefore offer new targets for chemotherapeutic treatment (Iorio *et al.*, 2005; Lu *et al.*, 2005; Scott *et al.*, 2006; Lowery *et al.*, 2009; Shenouda and Alahari, 2009).

The first evidence for the role of tumour suppressive miRNA in cancer came from studies involving the chromosomal deletion of 13q14 in B-cell chronic lymphocytic leucaemia (CLL). It was shown that this genomic region harbours the miR-15a and -16 genes. In addition, in those CLL patients without 13q14 deletion, miR-15a and -16 were frequently down-regulated (Calin *et al*., 2002). 13q14 chromosomal deletions also occurred in prostate cancer cell lines (Calin *et al.*, 2002). It has been illustrated that many miRNA are located at genomic fragile sites or loss-of-heterozygosity regions implicated in carcinogenesis (Calin *et al*., 2003; Scott *et al.*, 2006; Lu *et al.*, 2005). The polycistronic miR-17~92 was one of the first oncogenic miRNA identified (He *et al*., 2005; Olive *et al*., 2009; Wong *et al*., 2010), and up-regulation can increase cell proliferation *in vitro* and lymphoma in mice, dependent upon the MYC transcription factor. It is thought this is achieved through inhibiting $p21^{WAF1}$ expression, as the miR-17~92 gene harbours MREs in its 3' UTR (He *et al*., 2005; Olive *et al*., 2009; Wong *et al*., 2010).

It has also been reported that miRNA signatures between cancerous tissue and derived cell lines are altered from those of their normal counterparts. For instance, in breast and ovarian cancer, the up- or down-regulation of certain miRNAs were reported to associate with disease states as well as histopathological features. In ovarian cancer, these included the over-expression of miR-200a, -200c and -141, and the down-regulation of miR-145a, -140 and -125b (Iorio *et al*.,

2007). Moreover, nine miRNA, such as miR-10b, miR-125b and miR-21, were significantly induced or repressed in breast tumours. This differential expression pattern correlated between subtypes of breast cancer, such as hormone receptor-positive or -negative, vascular intrusive, proliferative index and lymph node metastatic (Iorio *et al.*, 2005). Furthermore, miR-645 and -410 were indicative of poor survival in advanced serous ovarian cancer (Shih *et al*., 2011).

In addition, it has been illustrated that altered epigenetic regulation can contribute to the down- or up-regulation of miRNA in cancer. As mentioned, sequencing has indicated that many miRNA promoters contain CpG islands, and those associated with hyper-methylation are frequently silenced (Lujambio *et al*., 2007; Tsai *et al*., 2011). In pancreatic cancer, promoter hyper-methylation of miR-132 interfered with Sp1 binding, and led to down-regulation of this tumour suppressive miRNA in cell lines. It was further shown that miR-132 induction could reduce proliferation and colony formation (Zhang *et al*., 2011), and methylation of miR-34b and miR-129-3p in cancerous cell lines exhibited reduced transcriptional activation, and were associated with poor prognosis in gastric cancers (Tsai *et al*., 2011). Studies have also identified Let-7 down-regulation in lung cancers (Saito *et al.*, 2006), and increased levels of miR-27a in breast cancer cell lines, the latter which led to induced expression of the Sp transcription factors, potentially driving oncogenic processes (Li *et al.*, 2010).

Among the oncogenic miRNA, miR-21 has been shown to be over-expressed in many cancers and able to induce tumorigenesis. Many studies have shown that suppressing miR-21 expression inhibits tumour growth and volume, as well as reduces cell proliferation and increases apoptosis *in vitro* (Si *et al*., 2007; Zhu *et al*., 2007; Lu *et al*., 2008). For instance, in the MCF-7 breast cancer cell line, exogenous induction of miR-21 increases cell proliferation, inhibits apoptosis and leads to transformation (Lu *et al*., 2008). While myriad mechanisms of miR-21-mediated tumorigenesis exist, it is likely that it is partially established through inhibiting translation of tumour suppressive genes, such as TMP1, Bcl-2 (Zhu *et al*., 2007), PTEN and PDCD4 (Lu *et al*., 2008).

In addition, expression of miRNAs from ERα-positive or -negative, and HER2/*neu* amplified cancer can predict prognosis. miRNAs such as miR-7, miR-128a, miR-210 and miR-516-3p have been shown to be associated with the aggressiveness of lymph node negative and ERα-positive breast cancer (Lowery *et al.*, 2009). Furthermore, miR-221/-222 is elevated in ERα-negative breast cancers and down-regulates expression of ERα mRNA through interaction

with the 3' UTR. Increased expression of miR-221/-222 in ERα-positive T47D and MCF-7 cell lines decreases the protein levels of ERα, but had no effect upon mRNA levels. It is thought that these miRNAs contribute to the progression of ERα-positive to ERα-negative breast cancers, and confer tamoxifen resistance in cell lines. Additionally, it was demonstrated that knockdown of miR-221/-222 in the ERα-negative cell line MDA-MB-468 restored ERα expression and tamoxifen sensitivity (Zhao *et al.*, 2008; Di Leva *et al*., 2010; Li *et al.*, 2010). It was further shown that ERα is able to bind to EREs in the 3' UTR of miR-221/-222 and these genes participate in a regulatory feedback loop (Di Leva *et al*., 2010).

In ERα-positive and -negative cell lines, miR-221/-222 and miR-206 have different cellular roles. In ERα-positive, miR-221/-222 cluster can increase proliferation, while in ERα-negative, it has the opposite effects and decreases cell growth. Moreover, miR-221/-222 induces apoptosis in ERα-negative cells. This potentially demonstrates the importance of determining the characteristics of breast cancer for targeted treatment (Di Leva *et al.*, 2010). ChIP studies have also indicated that ERα, in conjunction with retinoic acid receptors, binds to promoter regions in miR-23a and -210 (Saumet *et al*., 2012). Furthermore, miR-424 can be regulated by oestrogen in the ERα-positive MCF-7 cell line (Bhat-Nakshatri *et al*., 2009; Castellano *et al*., 2009; Saumet *et al*., 2012).

As previously mentioned, MYC induces transcription of the polycistronic miR-17~92, miRNAs which are frequently over-expressed in cancer (He *et al*., 2005; O'Donnel *et al*., 2005; Mu *et al*., 2009; Olive *et al*., 2009; Wong *et al*., 2010). It was further elucidated that miR-19b and -19a within this cluster are responsible for tumour formation in MYC-driven B-cell lymphoma mouse models, in part by suppressing apoptosis. Antago-miRs targeting the miR-17~92 cluster decreased tumour growth in the MYC-driven B-cell lymphoma mouse model, in part by targeting PTEN (Mu *et al*., 2009). The importance of MYC-driven miR-17~92 cluster has also been illustrated in ERα-positive breast cancer cell lines upon oestrogen treatment. Oestrogen-mediated miR-17~92 transcription, as well as transcription of the paralogous and polycistronic miR-106a~363, increased expression of the mature miR-18a, -19b and -20a within the clusters, leading to translational inhibition of ERα in a negative feedback loop. This occurred in a MYC-dependent manner, and interestingly, it was also shown that miR-17~92 could induce MYC expression by a feedback mechanism (Castellano *et al*., 2009).

There has also been extensive research into the therapeutic benefits of miRNA (Dyrskjot

et al., 2009; Yang *et al*., 2012). For example, in metastatic breast cancer, the expression of miR-34a and -34c are reduced compared to primary tumours, and over-expressing these tumour suppressive miRNA inhibited migration *in vitro* and distal pulmonary metastasis *in vivo*. It is thought inhibition of the oncogenic Fra-1 is responsible for miR-34a- and -34c-mediated anti-carcinogenesis (Yang *et al*., 2012).

Additional roles of miR-34 homologues (miR-34a, -34b and -34c) have been demonstrated in pancreatic cancer cells, where miR-34a expression is silenced through gene deletion and p53 suppression. p53-mediated transcription of the miR-34 family promotes apoptosis and p53-dependent changes in gene expression, such as those involved in DNA repair, anti-angiogenesis and apoptosis. Inducing miR-34a enhances p53-independent and -dependent apoptosis (Chang *et al*., 2007). Furthermore, over-expression of miR-129 arrested the cell cycle and induced cell death in bladder carcinoma cells, likely through down-regulation of the pro-apoptotic MCL1 protein (Mott *et al*., 2007; Dyrskjot *et al*., 2009), and miR-126 and -335 have been shown to suppress breast cancer metastasis (Tavazoie *et al*., 2008; Dyrskjot *et al*., 2009).

1.5.4.4 HDI-Mediated Regulation of miRNA Expression

Through the use of microarray techniques, it has been illustrated that miRNA expression is both tissue- and disease-specific, and that HDAC inhibitors can influence the expression profiles of miRNAs. It is suggested that targeting these tumour suppressive or oncogenic miRNA offers a new strategy for anti-cancer therapies (Scott *et al.*, 2006; Lu *et al.*, 2005).

Evidence for the role of aberrant epigenetic modifications in tumorigenesis comes from studies which disrupt DNA methylation patterns, leading to altered miRNA expression; for instance, hyper-methylation of CpG islands inactivates the expression of miR-124a (Lujambio *et al*., 2007). Additional studies have shown that the previously mentioned miR-15a and -16, as well as miR-29b, can be silenced by HDACs 1 through 3. Interestingly, those HDACs are over-expressed in approximately one-third of CLL patients with miR-15a and -16 silencing and deletion of chromosomal location 13q14 (Calin *et al*., 2002; Sampath *et al*., 2011). Furthermore, Entinostat and Vorinostat treatment increases expression of these miRNA, most likely through increased H3K4 methylation following increased histone acetylation (Sampath *et al*., 2011). Histone deacetylase inhibitor treatment also induced expression of miR-409 (Tsai *et al*., 2011), as

well as miR-127 (Saito *et al*., 2006; Tsai *et al*., 2011), which targets the 3' UTR of BCL6 (Saito *et al*., 2006). In addition, Entinostat has been shown to increase miR-125a, -125b and -205 expression in HER2-enriched cell lines, functioning synergistically to impair translation of HER2/*neu* and lead to apoptosis. The hydroxamates were unable to alter expression of these miRNA, and instead drug treatment led to reduced HER2/*neu* by different mechanisms, potentially dependent on reduced mRNA stability (Wang *et al*., 2013).

Studies have indicated that HDAC9 represses transcription of several pri-miRNA. While previous reports have implicated miR-18a, -19a and -19b in cancer proliferation, experimental evidence in mice models suggest that HDAC9 negatively regulates miR-17 and is involved in angiogenic processes. Inhibiting HDAC9 through 'pan-inhibitory' HDIs increased expression of the polycistron, and led to decreased vascular density and retinal vascularisation. It is interesting to note that polycistronic miRNA potentially play reciprocal roles in carcinogenesis, and HDI treatment can up-regulate only one mature miRNA in the cluster (Kaluza *et al*., 2013).

2. HYPOTHESIS AND SPECIFIC AIMS

Recent research completed in the Bonham Lab has demonstrated that the SRC and MYC proto-oncogenes are repressed in a variety of cancer-derived cell lines following HDI treatment. However, this observation was specific to 'pan-specific' inhibitors, such as TSA and NaB, as well as the class I-specific cyclic tetrapeptide Apicidin; the benzamides Entinostat and Mocetinostat had no effect on SRC and MYC expression levels. It is currently unknown whether this HDI-mediated repression is a global phenomenon occurring within all cancer-derived cell lines or is specific to certain cell types. In addition, the clinical significance of this repression in various molecular subtypes of breast cancer has yet to be determined.

Therefore, to investigate the repression of SRC and MYC in breast cancer-derived cell lines (Table 2.1), time course experiments were performed following treatment with various HDAC inhibitors (Table 2.2). It was hypothesised that while histone acetylation and $p21^{WAF1}$ expression are globally induced following drug treatment, HDI-mediated repression of SRC and MYC transcription is cell-specific, and thus not ubiquitious across the various cell lines due to the heterogenous nature of the cell lines. In addition, it was hypothesised that RNA pol II-transcribed miRNA are differentially regulated by these inhibitors in a manner similar to protein-coding genes.

Table 2.1. Molecular features and status for the ERα and PgR, and HER2/*neu* amplification in the selected breast cancer cell lines. These include the luminal A T47D (ERα- and PgR-positive), the mesenchymal Hs578T (triple-negative), the luminal B BT-474 (ERα- and PgR-positive with HER2/*neu* amplification) and the epithelial-like HCC-1419 (HER2-amplified) cell lines (Neve *et al*., 2006; Grigoriadis *et al*., 2012).

Cell Line	Subtype	ERα and PgR	HER2/*neu*
T47D	luminal A	present	normal
Hs578T	basal	absent	normal
BT-474	luminal B	present	amplified
HCC-1419	HER2/*neu*	absent	amplified

Table 2.2. Summary of the HDIs used in the experimental procedures and their associated specificity and chemical class. These include TSA (hydroxamate), Apicidin (cyclic tetrapeptide), and Entinostat and Mocetinostat (benzamides). While TSA is 'pan-specific', the other three HDIs are specific to class I HDACs.

Histone Deacetylase Inhibitor	Chemical Class	HDAC Specificity
Trichostatin A (TSA)	hydroxamate	pan-specific
Apicidin	cyclic tetrapeptide	class I
Entinostat (MS-275)	benzamide	class I
Mocetinostat (MGCD0103)	benzamide	class I

Rationale and Hypothesis: While HDIs are cytotoxic and capable of inducing both histone acetylation and $p21^{WAF1}$ expression, only certain classes exhibit the ability to repress SRC and MYC. Additionally, the class-specific and 'pan-inhibitory' HDIs can influence the expression of RNA pol II-transcribed tumour suppressor miRNA, which translates to miRNA-mediated effects on protein-coding genes. It is our hypothesis that certain classes of HDIs have an advantage over others due to their repressive capabilities on the proto-oncogenic SRC and MYC, and induction of potential tumour suppressors.

Specific Aims:

1. Determine the relative cytotoxicity and histone acetylation following treatment of 'pan-inhibitory' and class I-specific HDIs on four breast cancer cell lines representative of the molecular subtypes.

2. Determine the effects of 'pan-inhibitory' and class I-specific HDIs treatment upon the relative induction of $p21^{WAF1}$, and repression of proto-oncogenes SRC and MYC.

3. Determine the effects of 'pan-inhibitory' and class I-specific HDIs on mature miRNA, and examine the protein and mRNA levels of potential mRNA targets in both HDI-treated and miRNA transfected cells.

3. MATERIALS AND METHODS

3.1 Reagents, Equipment, Software and Distributors

The distributors of the reagents and commercially available kits used in the following procedures are listed in Table 3.1 and Table 3.2, respectively. The equipment and software programmes that have been used to analyse data are correspondingly outlined in Table 3.3 and Table 3.4. The forward and reverse primer sequences for the expression of protein-coding genes, purchased from Invitrogen, are reported in Table 3.5 with their annealing temperatures in Table 3.6. The gene-specific primer assay kits for miRNA were purchased from Qiagen with catalogue number and the mature miRNA sequence listed in Table 3.7. The synthetic miRNA mimics used in transfection experiments were purchased from Qiagen and are listed with their catalogue numbers in Table 3.8. The antibodies and dilutions used in immuno-blotting experiments are reported in Table 3.9.

3.2 Cell Lines and Tissue Culture

3.2.1 Culture and Standard Maintenance of Cell Lines

The human breast cancer cell lines utilised in this study were purchased from the American Type Culture Collection (ATCC), and the cell media used to propagate the cell lines were purchased from Invitrogen-Gibco Cell Culture Systems. The metastatic-derived T47D mammary ductal carcinoma cell line was cultured in RMPI-1640 media, supplemented with insulin; the Hs578T mammary carcinoma cell line was cultured in DMEM media, with 0.01 mg/mL insulin; the BT-474 ductal mammary carcinoma cell line was cultured in DMEM; the HCC-1419 primary ductal carcinoma cell line was cultured in RMPI-1640. The media contained 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin, and the cell lines were grown in an incubator at 37 ^{O}C and with a 5% CO_2 atmosphere. These breast cancer cell lines were chosen due to their reported classification (Neve *et al.*, 2006; Grigoriadis *et al.*, 2012), and no verification was performed in our laboratory. Upon propagation and subsequent sub-culturing, Trypsin-EDTA were used to displace the cells from their tissue culture plates.

Table 3.1. The supplier addresses.

Reagent Supplier	Location
Abcam	Cambridge, MA. USA
American Type Culture Collection (ATCC)	Manassas, VA, USA
Applied Biosystems Canada	Streetsville, ON, Canada
Bio-Rad Laboratories, Ltd.	Mississauga, ON, Canada
Cedarlane Laboratories	Burlington, Ontario, Canada
EMD Chemicals	Gibbstown, NJ, USA
Fermentas Canada, Inc.	Burlington, ON, Canada
Fisher Scientific	Ottawa, ON, Canada
Gibco Life Technologies	Burlington, ON, Canada
Invitrogen Canada, Inc.	Burlington, ON, Canada
LICOR Technologies	Lincoln, NB, USA
Millipore	Billerica, MA, USA
Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Sigma-Aldrich, Co.	Oakville, ON, Canada
Thermo Scientific	Rochester, NY, USA
Qiagen, Inc.	Mississauga, ON, Canada
VWR	Mississauga, ON, Canada

Table 3.2. Suppliers of commercially available kits.

Commericially Available Kits	Supplier
miRNeasy Mini Kit	Qiagen, Inc.
iScript cDNA Synthesis Kit	Bio-Rad Laboratories, Ltd.
miScript RT II Kit	Qiagen, Inc.
miScript SybrGreen PCR Kit	Qiagen, Inc.
Total Protein, Micro Lowry Kit	Sigma-Aldrich, Co.
SsoFast EvaGreen Supermix	Bio-Rad Laboratories, Ltd.

Table 3.3. The suppliers for the equipment used.

Equipment	Suppliers
27 gauge precision glide syringe	BD Biosciences
AccuPower Model 300 Power Supply	VWR
Analogue Vortex Mixer	VWR
Biofuge 13 Heraeus	Baxter CanLab
GeneAmpPCR 2720 Thermal Cycler	Applied Biosystems Canada
LICOR Odyssey Visualisation System	LICOR Technologies
MicroMax Centrifuge	IFC
Mini Protean II cells	Bio-Rad Laboratories, Ltd.
NanoDrop 2000c Spectrophotometer	Thermo Scientific
OWL Electroblotting Unit	Thermo Scientific
Quickseal	SA Equipment
SmartSpec 3000 Spectrophotometer	Bio-Rad Laboratories, Ltd.
Standard Heatblock	VWR
StepOnePlus Real Time PCR System	Applied Biosystems Canada
UV Transilluminator	Bio-Rad Laboratories, Ltd.

Table 3.4. The suppliers for the software programmes used.

Software Programmes	Suppliers
Primer Express Version 3.0	Applied Biosystems Canada
Licor Odyssey Imaging System	LICOR Technologies
OneStepPlus Real Time PCR System	Applied Biosystems

Table 3.5. Forward and reverse primer sequences, purchased from Invitrogen.

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
$p21^{WAF1}$	GCCTGCCGCCGCCTCTTC	GCCGCCTGCCTCCTCCCAACTC
SRC	CAGAGGAGCCCATTTACATCGTC	CCCTTGAGAAAGTCCAGCAAACTC
MYC	GGACTTGTTGCGGAAACGA	TTACGCACAAGAGTTCCGTAGCT
RPL13A	CAAGGTGTTTGACGGCATCC	GCTTTCTCTTTCCTCTTCTCCTCC
CCND1	GCATGTTCGTGGCCTCTAAGA	CGGTGTAGATGCACAGCTTCTC
c-MYB	CCCACTGTTAACAACGACTATTCCT	GGTATGGAACATGACTGGAGACATT
CHEK1	GAGTACCGCACTCTGAGGTTTACA	CCACCGGACAGGACTGTGA
ERα	TCTGCCAAGGAGACTCGCTACT	CACAGGACCAGACTCCATAATGG

Table 3.6. Annealing temperatures for the RT-qPCR primer sets.

Primer Set	Annealing Temperature
SRC	55 ^{O}C
MYC	60 ^{O}C
$p21^{WAF1}$	60 ^{O}C
c-Myb	59 ^{O}C
ERα	59 ^{O}C
CCND1	60 ^{O}C
CHEK1	59 ^{O}C

Table 3.7. miScript Primer Assays obtained from Qiagen.

miRNA	Assay Name	Mature miRNA Sequence	Catalogue Number
miR-129-5p	Hs_miR-129_1	CUUUUUGCGGUCUGGGCUUGC	MS00006643
miR-424	Hs_miR-424_1	CAGCAGCAAUUCAUGUUUUGAA	MS00004186
miR-9-3p	Hs_miR-9*_1	AUAAAGCUAGAUAACCGAAAGU	MS00006510
RNU6	Hs_RNU6-2_11		MS00033740

Table 3.8. miScript miRNA Mimics obtained from Qiagen.

miRNA	Assay Name	Catalogue Number	Guide Strand (Mature miRNA)
miR-129-5p	Syn-hsa-miR-129-5p	MSY0000242	CUUUUUGCGGUCUGGGCUUGC
miR-424	Syn-hsa-miR-424-5p	MSY0001341	CAGCAGCAAAACAUGUUUUGAA
miR-9-3p	Syn-hsa-miR-9*	MSY0000442	AUAAAGCUAGAUAACCGAAAGU
miR-1	Syn-has-miR-1	MSY0000416	UGGAAUGUAAAGAAGUAUGUAU

Table 3.9. Antibodies used in immuno-blotting experiments.

Antibody	Company	Cat No.
Chek1	Santa Cruz Biotechnology	sc-377231
HDAC5	Abcam	ab11969
Cyclin D1	Abcam	ab6152
ERα	Abcam	ab75635
Histone H3Ac (K9, K18, K23, K27)	Abcam	ab47915
Histone H3K9Ac	Abcam	ab4441
Histone H3	Abcam	ab1791
GAPDH	Abcam	ab8245

3.2.2 Histone Deacetylase Inhibitor Treatments

Prior to drug treatments, all cell lines were trypsinised, sub-cultured and grown for 36-48 hours to 70-75% confluency.

3.2.2.1 Time Course Experiments

The media were aspirated and the T47D, Hs578T, BT-474 and HCC-1419 cell lines were grown over 24 hr in fresh media containing 1 µM TSA, 2 µM Apicidin, 2 µM Entinostat or 1 µM Mocetinostat. The Hs578T cell lines were also treated with 2 µM TSA, 5 µM Apicidin, 5 µM Entinostat or 5 µM Mocetinostat over 24 hr. The cells were harvested for RNA in Qiazol™ Lysis Buffer or protein in 2X Laemmli sample buffer (10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 2% [w/v] SDS, 65 mM Tris-HCl [pH 7.0] and 0.05% [w/v] bromophenol blue) at subsequent time intervals of 1 hr, 3 hr, 6 hr, 12 hr and 24 hr. Ethanol (EtOH) and dimethyl sulphoxide (DMSO) drug vehicle controls and untreated controls were also harvested at this time.

3.2.2.2 Dose Response Experiments

The media were aspirated and the T47D, Hs578T, BT-474 and HCC-1419 cell lines were grown in fresh media containing 0.25 µM, 0.5 µM, 1 µM, 2 µM or 5 µM TSA, Apicidin, Entinostat or Mocetinostat. The cells were harvested for protein 2X Laemmli sample buffer (10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 2% [w/v] SDS, 65 mM Tris-HCl [pH 7.0] and 0.05% [w/v] bromophenol blue) after 12 hr; EtOH and DMSO drug vehicle controls and untreated controls were also harvested.

3.2.3 miRNA Mimic Transfection Experiments

The Hs578T and T47D cells were seeded at 1×10^4 or 2×10^4 cells in 24- or 12-well plates, respectively, and allowed to grow for 24-36 hr prior to transfections. 300 nM synthetic miRNA Mimics purchased from Qiagen (miR-424, miR-19-5p or miR-9-3p) were incubated with Lipofectamine 2000 for 4h, followed by the addition of an equal amount of DMEM or RPMI-

1640 media containing 20% FBS. RNA and/or protein were harvested as previously described over 6 hr, 12 hr, 24 hr, 36 hr, 48 hr or 72 hr. Untreated, mock, scrambled and miR-1 controls were also harvested at this time.

3.3 Real Time Polymerase Chain Reaction

3.3.1 Total RNA Purification from Cultured Cells

Total RNA, including small non-coding RNAs and mRNA, were isolated utilising Qiagen's miRNeasy Mini Kit. Following HDI treatments, cells were harvested directly from the plate in 700 µL of Qiazol™ Lysis Buffer and homogenised by passing through a 4 gauge-syringe. The sample lysates were stored at -80 ^{O}C until total RNA was to be extracted. The lysates were thawed to room temperature and incubated for 5 min prior to the addition of 140 µL chloroform and subsequent vortexing. Following centrifugation at 12,000 x g for 15 min, the aqueous phase was passed through a miRNeasy Mini column and treated with 1 unit DNase-I for 15 min at room temperature to digest contaminating genomic DNA. The sample RNA was then purified according to the manufacturer's protocol. The concentration of the RNA was determined by measuring the absorbance at 260 nm on a SmartSpec 3000 Spectrophotometer or a NanoDrop 2000c Spectrophotometer, with the A_{260} reading equal to 1 = 40 µg/mL RNA. The purification and quality of the RNA was identified by an A_{260}:A_{280} ratio between 1.6 and 2.0, and the RNA quality was further assessed by agarose gel electrophoresis, with visual assessment of the 28S and 18S ribosomal subunits by ethidium bromide fluorescence.

3.3.2 mRNA Procedures

3.3.2.1 Synthesise of Complementary DNA

Following purification and quality determination, 1 µg of total RNA was used to synthesise complementary DNA (cDNA), according to Bio-Rad's iScript reverse transcriptase procedure. cDNA amplification was performed in a GeneAmpPCR System 2700, programmed

to the following protocol: 5 min at 25 ^{O}C, 30 min at 42 ^{O}C and 5 min at 85 ^{O}C. cDNA was stored at -20 ^{O}C until required.

3.3.2.2 Real Time PCR for mRNA Quantification

Real Time PCR was performed to analyse mRNA expression following HDI-treatment or miRNA transfection. Reactions were set up with 0.3 µL cDNA to a final volume of 10 µL with 5 µL QuantiFast SSO SybrGreen and 66 ng of gene-specific primers designed with Primer Express® version 3.0 software purchased from Applied Biosystems, or primers previously designed and tested in the Bonham Lab (Table 3.5). Triplicate reactions were performed on an Applied Biosystems StepOnePlus Real Time machine, according to the manufacturer's instructions and analysed with StepOne™ Software v2.2.2. The PCR cycling programme was set for an initial 20 sec at 95 ^{O}C followed by 40 cycles of 95 ^{O}C for 3 sec and an appropriate primer-specific annealing temperature for 30 sec (Table 3.9). A melting curve was performed following amplification of the Real Time assays and product specificity was assessed by calculating the melting temperature of the amplicon. The samples were analysed in at least two technical repeats in three biological repeats, and the fold changes were calculated by the ΔΔCt method using RPL13A as an internal control.

3.3.3 microRNA Procedures

3.3.3.1 Synthesise of Complementary DNA

cDNA was synthesised from 2 µg total RNA according to the manufacturer's protocol in the miScript RT II kit acquired from Qiagen. cDNA amplification was completed in a GeneAmpPCR System 2700 set to the following protocol: 25 ^{O}C for 5 min, 37 ^{O}C for 60 min and 85 ^{O}C for 5 min. The cDNA was stored at -20 ^{O}C and diluted 1:10 with sterilised water prior to use.

3.3.3.2 Real Time PCR for miRNA Quantification

Real Time PCR was carried out with 2.5 µL cDNA per reaction, 2.5 µL 1X universal miScript primer, 2.5 µL 1X miRNA-specific primer (Table 3.6) and 12.5 µL SybrGreen in a final volume of 25 µL. Reactions were performed in triplicate on an Applied Biosystems StepOnePlus Real Time machine according to the manufacturer's instructions and analysed with StepOne™ Software v2.2.2. The PCR cycling programme was run at 95 ^{O}C for 15 min, followed by 40 cycles at 15 sec at 94 ^{O}C, 30 sec at 55 ^{O}C and 30 sec at 70 ^{O}C. The fold changes were calculated by the ΔΔCt method using RNU6 as an internal control and the samples were analysed in at least three technical repeats in three biological repeats. A melting curve was performed following amplification of the Real Time assays to assess product specificity. In addition, a no-template control and a positive miRNA control were also included in the Real Time experiments to assess possible false-positive amplifications. Products were commonly subjected to electrophoresis on a 3% agarose gel.

3.4 Cytotoxic Assays

The anti-proliferative and cytotoxic activities of the HDIs were determined by the MTT assay. The T47D, Hs578T, BT-474 and HCC-1419 cells were seeded in 96-well plates at a concentration of 1 x 10^4 cells and allowed to grow under normal growth conditions for 36-48 hours. The cells were subsequently treated for 48h with 0.01 µM, 0.1 µM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM of TSA, Apicidin, Entinostat or Mocetinostat. Untreated, and EtOH and DMSO vehicle controls were also included at a concentration equal to that in the drug-treated cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added at a final 0.5 mg/mL concentration and incubated for 4h at 37 ^{O}C, and the formed formazan crystals were solubilised with isopropanol alcohol and 0.4 N HCl. The absorbance at wavelengths 540 nm and 630 nm were recorded on a SpectraMax ELISA plate reader. The experiments were repeated in triplicate for three independent assays and the cell viability calculated compared to untreated control. The concentration required to inhibit 50% cellular growth (IC_{50}) was obtained by visual inspection of the viability vs HDI concentration graphs.

3.5 Western Blot

3.5.1 Protein Isolation from Cultured Cells and Lowry Protein Assay

Following HDI treatments, cells were harvested directly from the plate in 2X Laemmli sample buffer containing 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 65 mM Tris-HCl (pH 7.0) and 0.05% (w/v) bromophenol blue. Samples were boiled for 5 min and homogenised by passing through a 27-gauge syringe. Protein concentrations were calculated utilising a Total Protein, Micro Lowry Kit purchased from Sigma-Aldrich, according to the manufacturer's protocol. The absorbances at 750 nm were read on a SmartSpec 3000 Spectrophotometer and the concentrations were determined alongside a standard curve.

3.5.2 Immuno-Blot Procedure

3.5.2.1 SDS-PAGE Gel Electrophoresis

Protein samples were subjected to electrophoresis on 10% or 15% polyacrylamide resolving gels (29.2% acrylamide to 0.8% bis-acrylamide, 375 mM Tris-HCl [pH 8.8], 0.1% [v/v] SDS, 0.1% [w/v] ammonium persulfate and 0.04% [w/v] N,N,N',N'-tetramethylethylenediamine [TEMED]) and a 5% polyacrylamide stacking gel (29.2% acrylamide to 0.8% bis-acrylamide, 130 mM Tris-HCl, 0.1% [w/v] SDS, 0.1% [w/v] ammonium persulfate and 0.04% [w/v] TEMED) (Sambrook *et al.*, 1989). Prior to electrophoresis, protein samples were boiled for 5 min at 100 ^{O}C and 25 µg protein resolved on the SDS-PAGE gels. Procedures were performed with the Mini Protean II cells at 140-160V for approximately 90-120 min.

3.5.2.2 Nitrocellulose Membrane Transfer

The gels were saturated with transfer buffer containing 10 mM Tris, 15 mM NaCl, 0.5% (v/v) TWEEN-20 for 15 min. The resolved proteins were transferred by semi-dry transfer to a nitrocellulose membrane for 15 min at 400 mA, utilising an OWL electroblotting unit. Protein

transfer was confirmed by Ponceau-S (0.1% [w/v] Ponceau-S in 5% [v/v] acetic acid) staining, followed by stain removal with distilled water and 1X PBS (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, pH 7.4). The membranes were then blocked for 1 hr in one part Odyssey blocking buffer and one part PBS.

3.5.2.3 Antibodies

Primary antibodies were incubated overnight at 4 ^{O}C following by washing in 1X PBST (PBS and 0.5% TWEEN-20) and secondary antibody incubation for 1 hr at room temperature, followed by 1X PBST and 1X PBS washes. The primary antibodies, catalogue numbers and dilutions in Odyssey blocking buffer with 0.05% TWEEN-20 are listed in Table 3.8. The goat secondary antibodies were labelled with IR680 or IR800 dye, and incubated at a 1:30,000 dilution in Odyssey blocking buffer and 0.05% TWEEN-20. The membranes were visualised using fluorescence on a LICOR Odyssey visualisation system and associated software. In certain cases, the membranes were stripped for 5 min in Licor Odyssey stripping buffer and three parts PBS, prior to being re-probed overnight in a different primary antibody. In all immuno-blot assays, the total protein levels of histone H3 and/or GAPDH were assessed as loading controls and to further demonstrate that total H3 levels remained static in acetylation experiments.

3.6 Statistical Analysis

All statistical analyses were performed using the SigmaStat software version 12.2, with a p-value equal to or less than 0.05 considered to be statistically significant. The p-values were based on a one-way analysis of variance (ANOVA) by the Holm-Sidak method (Sidak, 1967; Holm, 1979; Seaman *et al*., 1991; Aickin and Gensler, 1996), and the represented graphs are the mean of at least two technical repeats in three independent experiments, +/- SEM. Statistically significant values compared to untreated controls are indicated by asterisks.

4. RESULTS

4.1 The Effect of Histone Deacetylase Inhibitors on Breast Cancer Cell Viability, Histone H3 Acetylation, and $p21^{WAF1}$ Induction

Class- and isoform-specific HDIs have been shown to exhibit cytotoxic, apoptotic and/or anti-proliferative properties in a variety of human cancer-derived cell lines. In addition, treatment leads to increased acetylation of histone and non-histone proteins, eliciting cellular processes such as induction of the cell cycle inhibitor $p21^{WAF1}$ (Vigushin *et al*., 2001; Glaser *et al*., 2003; Beckers *et al*., 2007; Ueda *et al*., 2007; Im *et al*., 2008; Fournel *et al*., 2008; Khan *et al*., 2008; Ahn *et al*., 2009; Kelly and Cowley, 2013). Nonetheless, while the cellular effects of class-specific drugs can be similar, they have also demonstrated diversity in the subset of genes targeted and in their anti-proliferative or cytotoxic properties. In addition, apoptotic pathways can be activated in diverse ways through the activation of different pro-apoptotic pathways. It is thought that this diversity is partially responsible for the differential cellular effects observed within certain cell lines (Chatterjee *et al*., 2013).

Therefore, the effects of four structurally-diverse HDIs on cell viability, histone acetylation and gene expression were investigated in four cell lines representative of the heterogenous nature of breast cancer. These cell lines were chosen based on their status of the oestrogen receptor-α (ERα), progesterone receptor (PgR) and HER2/*neu* amplification, and included the ERα- and PgR-positive T47D cell line, the triple-negative Hs578T cell line, the ERα- and PgR-positive and HER2-amplified BT-474 cell line, and the HER2-amplified HCC-1419 cell line (Neve *et al*., 2006; Grigoriadis *et al*., 2012). Additionally, the selection of HDIs used in this study were based upon their class-specificity and diverse chemical structures; these included the hydoxamic acid TSA, the cyclic tetrapeptide Apicidin, and the benzamides, Entinostat and Mocetinostat.

4.1.1 The Cytotoxic Influence of Histone Deacetylase Inhibitors in Breast Cancer Cell Lines

To determine the anti-proliferative properties these compounds have on the various breast cancer-derived cell lines, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

assays were performed. The cell lines were then analysed for cellular viability following 48h administration with increasing concentrations of the compounds. The assays were performed in triplicate in at least three independent experiments, and were completed by Kyoo Yoon Choi, an honours project student under my supervision.

These HDIs exhibited anti-proliferative properties in the four breast cancer cell lines in a dose-dependent manner (Figure 4.1 and 4.2). In general, the cell lines had reduced viability following HDI treatment; however, the range of concentrations indicated that HDI-mediated cytotoxicity varied between the cell lines (Table 4.1). The IC_{50} values for the T47D cell line were calculated to be 0.20 µM (TSA), 0.25 µM (Apicidin), 2.5 µM (Entinostat) and 2.5 µM (Mocetinostat). TSA and the benzamides were less toxic to the Hs578T cell lines, with IC_{50} values of 2.2 µM (TSA), 0.2 µM (Apicidin), 2.5 µM (Entinostat) and 202 µM (Mocetinostat). TSA and Apicidin exhibited greater cytotoxicity than the benzamides in the BT-474 cell line; the IC_{50} values were 0.2 µM (TSA), 0.25 µM (Apicidin), 2.2 µM (Entinostat) and 5.0 µM (Mocetinostat). Entinostat had less effect in the HCC-1419 cell line, and the IC_{50} values were calculated to be 0.4 µM (TSA), 0.3 µM (Apicidin), 25 µM (Entinostat) and 12 µM (Mocetinostat).

Apicidin treatment resulted in similar cytotoxic profiles, whereas TSA and the benzamides exhibited a greater range of cytotoxic effects in the cell lines. TSA resulted in significantly reduced viability with low IC_{50} concentrations, with the exception of the Hs578T cells. Generally, the benzamides possessed the lowest cytotoxicity of the tested compounds, with BT-474 and HCC-1419 cell lines being less sensitive to Mocetinostat and Entinostat, respectively. Thus, it was observed that HDI-treatment resulted in reduced cell viability in a dose-dependent manner.

4.1.2 Dose-Dependent and Time-Dependent Histone H3 Acetylation Following Histone Deacetylase Inhibitor Treatment

To assess the extent of HDI-mediated histone acetylation, immuno-blotting for acetylated histone H3 (H3Ac) was performed. Dose responses were used to compare concentration-dependent HDI-mediated histone acetylation in the cell lines. In addition, treatments were performed over 24 hr to determine the induction of histone H3 lysine 9 (H3K9) acetylation

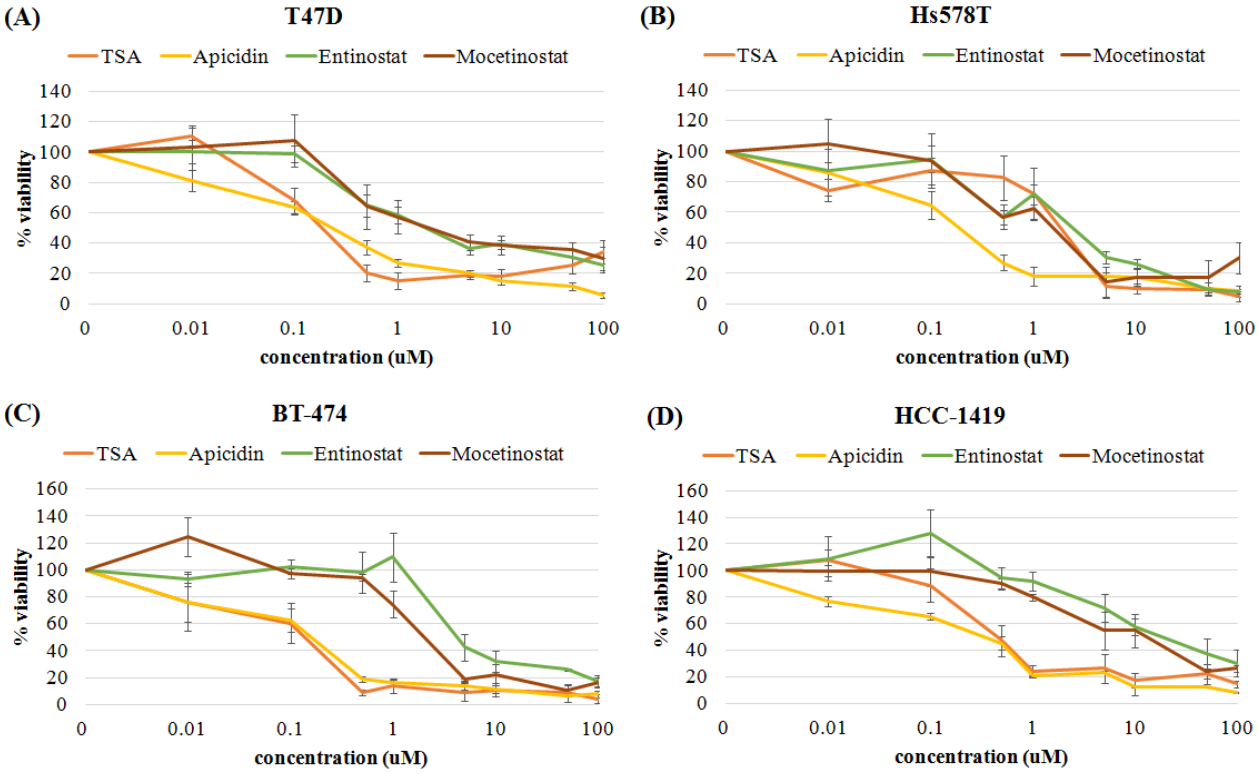

Figure 4.1. Decreased viability of breast cancer cells following HDI treatment. As indicated, 10^4 (A) T47D, (B) Hs578T, (C) BT-474 or (D) HCC-1419 were seeded in 96-well plates and treated with 0.01 µM, 0.1 µM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM TSA, Apicidin, Entinostat or Mocetinostat for 48 hr. The cell viability was assessed by the MTT assay. Data shown are the average cell viability from three independent experiments, +/- SEM.

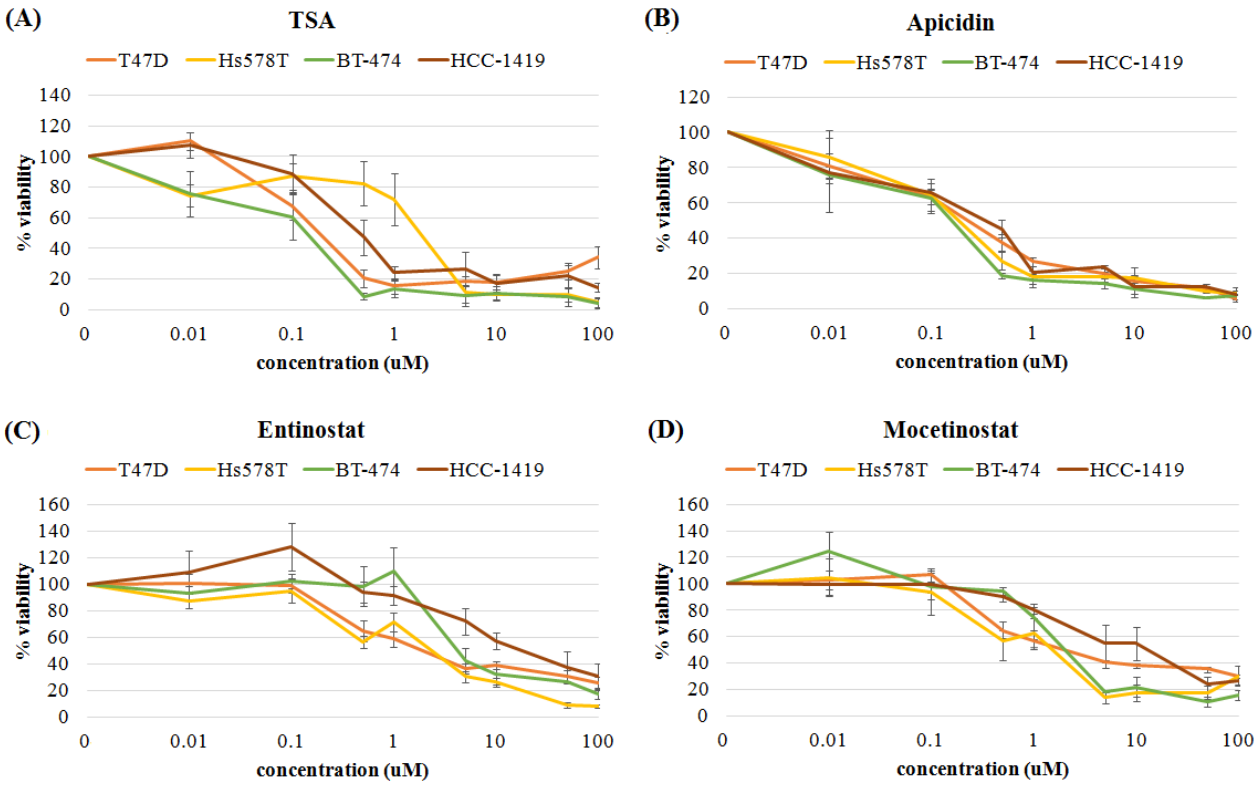

Figure 4.2. Histone deacetylase inhibitors result in decreased cell viability in breast cancer cells. 10^4 cells were seeded in 96-well plates and treated with 0.01 µM, 0.1 µM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM (A) TSA, (B) Apicidin, (C) Entinostat or (D) Mocetinostat for 48 hr. The cell viability was assessed by the MTT assay. Data shown are the average cell viability from three independent experiments, +/- SEM.

Table 4.1. IC_{50} values from HDI-treated breast cancer cell lines.

	TSA (μM)	Apicidin (μM)	Entinostat (μM)	Mocetinostat (μM)
T47D	0.2	0.25	2.5	2.5
Hs578T	2.2	0.2	2.5	2.0
BT-474	0.2	0.2	2.5	5.0
HCC-1419	0.4	0.3	25	12

(H3K9Ac) over time. The immuno-blotting procedures were repeated in two independent experiments; each assay exhibited consistent results. The data in the forthcoming section is representative of these results.

HDI treatment increased histone H3 acetylation in a dose-dependent manner in the four cell lines tested (Figure 4.3). While the higher drug concentrations enhanced acetylation to a greater extent, certain HDIs were less effective. The T47D, BT-474 and HCC-1419 cell lines were highly responsive to HDI administration, with acetylation induced at the 0.25 µM dosage. However, in the Hs578T cell line, the 2 µM TSA, 5 µM Entinostat and 5 µM Mocetinostat concentrations led to only modest H3 acetylation. Furthermore, the benzamides had similar profiles in each cell line and gradually induced histone acetylation in a dose-dependent manner.

Based on these observations, the concentrations for further experiments involving the Hs578T cell line were 2 µM TSA and 5 µM Apicidin, Entinostat and Mocetinostat. The T47D, BT-474 and HCC-1419 cell lines were treated with 1 µM TSA, 2 µM Apicidin, 2 µM Entinostat and 1 µM Mocetinostat. These reflect the enhanced histone acetylation observed, and confirms that the cell lines are treated with HDI concentrations that induce similar levels of histone acetylation. Therefore, any differences observed between the cell lines due to inconsistencies in the HDI administration can be eliminated.

In addition to these dose-dependent observations, immuno-blotting against H3K9 illustrated that acetylation increased in a time-dependent manner upon HDI administration in the cell lines (Figure 4.4). TSA treatment in the T47D cell line increased acetylation after 3 hr treatment, while Apicidin, Entinostat and Mocetinostat lead to acetylation at 12 hr. These effects were still observed following 24 hr treatment. The Hs578T and BT-474 cells were less responsive to class I-specific HDIs and acetylation increased after 6 hr to 12 hr treatment, whereas TSA enhanced H3K9 acetylation as early as 3 hr. While histone acetylation mediated through TSA treatment exhibited transient properties within the Hs578T, BT-474 and HCC-1419 cell lines, with its effects eliminated 24 hr after treatment, the class I-specific HDIs sustained enhanced acetylation over the 24 hr period. HDI treatment in the HCC-1419 cell line induced acetylation as early as 1 hr treatment. Similarly, the effects of TSA were transient. These experiments were also repeated with a pan-acetylated H3 antibody and gave similar results, and as was expected, the levels of total H3 and/or GAPDH remained unaltered throughout the treatments.

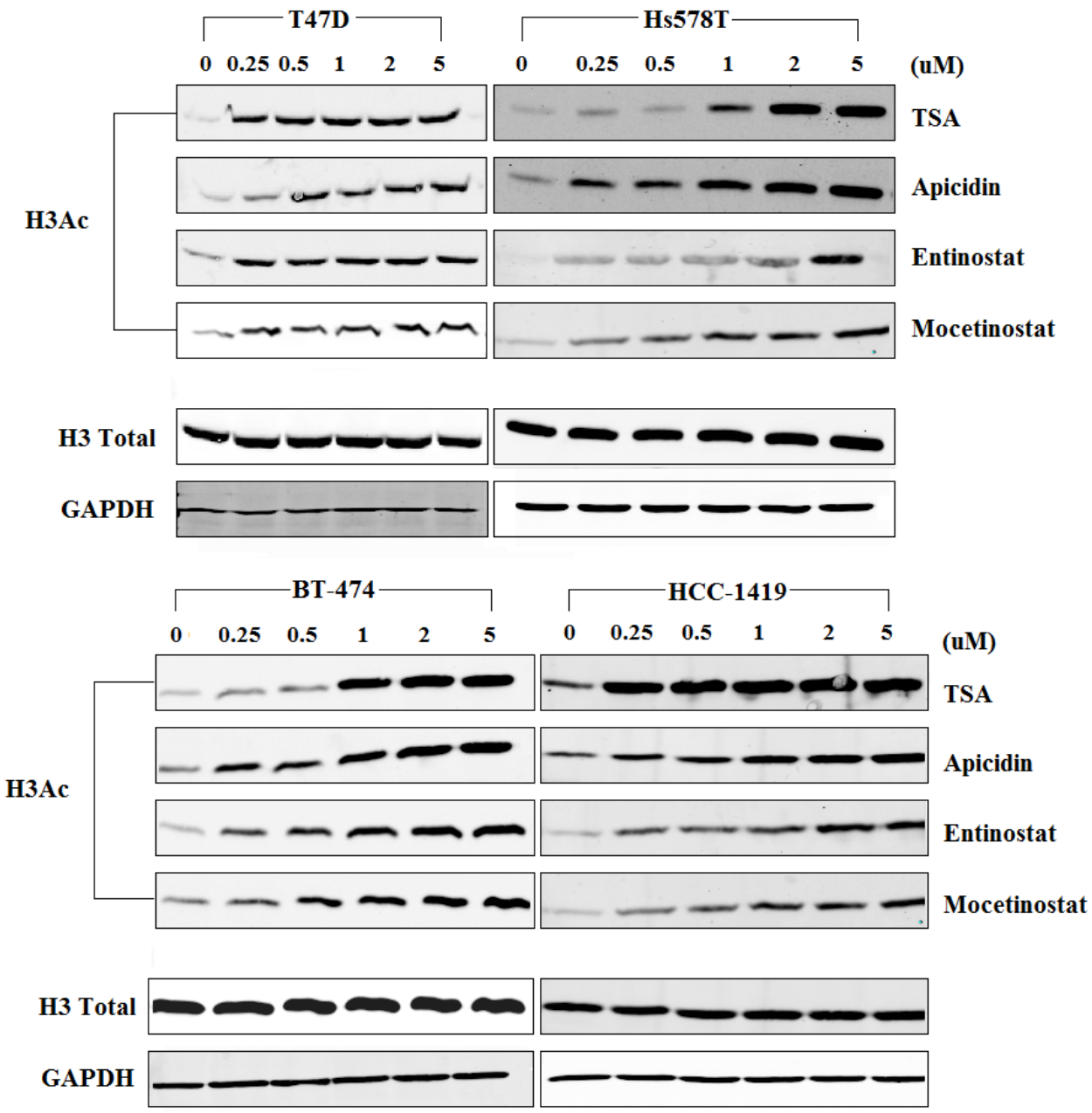

Figure 4.3. Increased histone H3 acetylation following HDI treatment. T47D, Hs578T, BT-474 or HCC-1419 cells were treated for 12 hr with the indicated concentrations of TSA, Apicidin, Entinostat or Mocetinostat. Treated cells were harvested in 2X Laemmli sample buffer and 25 µg protein lysate was resolved via SDS-PAGE, followed by immuno-blotting against acetylated H3 (H3Ac), total H3 and GAPDH as a loading control. The data shown is representative of two biological repeats, which gave similar results.

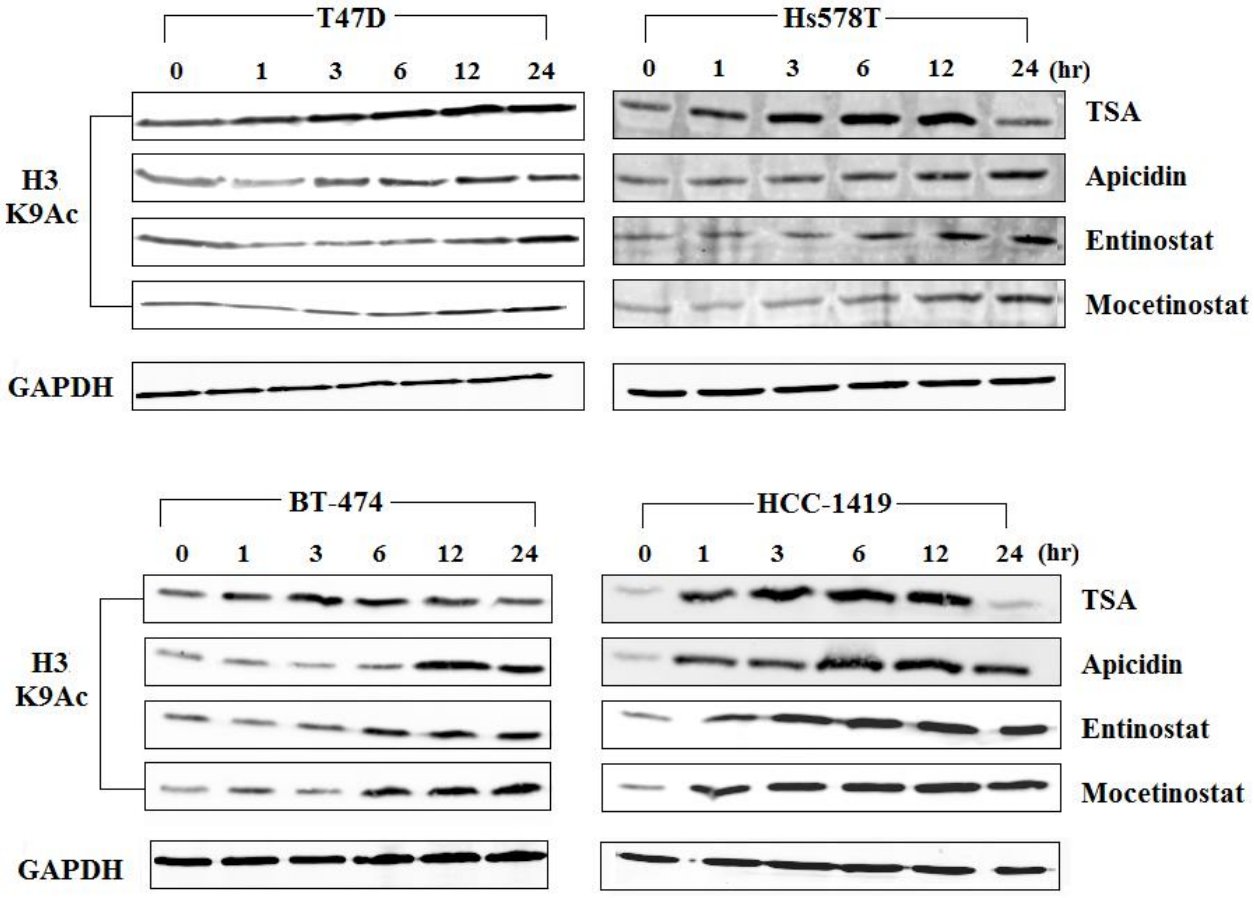

Figure 4.4. Increased H3K9 acetylation upon HDI treatment. T47D, Hs578T, BT-474 and HCC-1419 cells were treated with 1 µM TSA, 2 µM Apicidin, 2 µM Entinostat or 1 µM Mocetinostat at the indicated time points. Cells were harvested in 2X Laemmli sample buffer and 25 µg protein resolved via SDS-PAGE. Immuno-blotting against acetylated lysine 9 of H3 (H3K9Ac) and GAPDH as a loading control was performed. The data shown is representative of two biological repeats, which gave similar results.

Therefore, in addition to their anti-proliferative properties, the three chemical classes of HDIs enhance histone acetylation in both a dose-dependent and time-dependent manner. Minimal cell-specific responses were observed between the diverse compounds, highlighted with the transient nature of TSA in the Hs578T and HCC-1419 cell lines and the low responsiveness of the benzamides in the Hs578T cell line. However, they generally act to enhance histone H3 acetylation within the four cell lines.

4.1.3 Histone Deacetylase Inhibitors Induce $p21^{WAF1}$ Expression

It has been extensively reported in the literature that $p21^{WAF1}$ is universally up-regulated following HDI treatment in numerous cancer-derived cell lines (Ocker and Scheider-Stock, 2007; Gartel and Tyner, 2002). In addition, studies performed in the Bonham Lab have shown that $p21^{WAF1}$ expression is generally up-regulated following TSA and NaB treatment in colorectal, hepatocarcinoma and breast cancer cell lines (Hirsch and Bonham, 2004). Therefore, HDI-mediated $p21^{WAF1}$ induction was assessed in the four cell lines to set a baseline to compare differential effects of these drugs on proto-oncogene expression, in addition to verify the activity of compounds. Following an HDI time course, reverse transcriptase quantitative PCR (RT-qPCR) was performed with $p21^{WAF1}$-specific primers.

Induction of $p21^{WAF1}$ mRNA was observed in all four cell lines after HDI treatment (Figure 4.5). However, the degree and duration of $p21^{WAF1}$ expression varied significantly between them, indicating cell-specific responses. TSA (1 µM) induced $p21^{WAF1}$ expression 8.01 fold ($p<0.001$) in the T47D cell line, while 2 µM Apicidin, 2 µM Entinostat and 1 µM Mocetinostat increased expression 3.97 fold ($p<0.001$), 5.03 fold ($p<0.001$) and 3.09 fold ($p<0.001$), respectively. The effects of TSA and Apicidin were transient and $p21^{WAF1}$ expression returned to control levels after 24 hr treatment.

TSA (2 µM) administration resulted in the greatest induction in the Hs578T cell line, with an 11.21 fold change ($p<0.001$) after 12 hr treatment. Apicidin (5 µM) resulted in a 4.68 fold ($p<0.01$) induction at 6 hr, while Entinostat and Mocetinostat (5 µM) induced expression 5.45 fold ($p<0.001$) and 5.14 fold ($p<0.001$) at 24 hr, respectively. The up-regulation of $p21^{WAF1}$ was moderate in the Hs578T cells treated with 2 µM TSA and 5 µM Apicidin, Entinostat or Mocetinostat, although treatment with the lower dosages used in the three additional cell lines (1

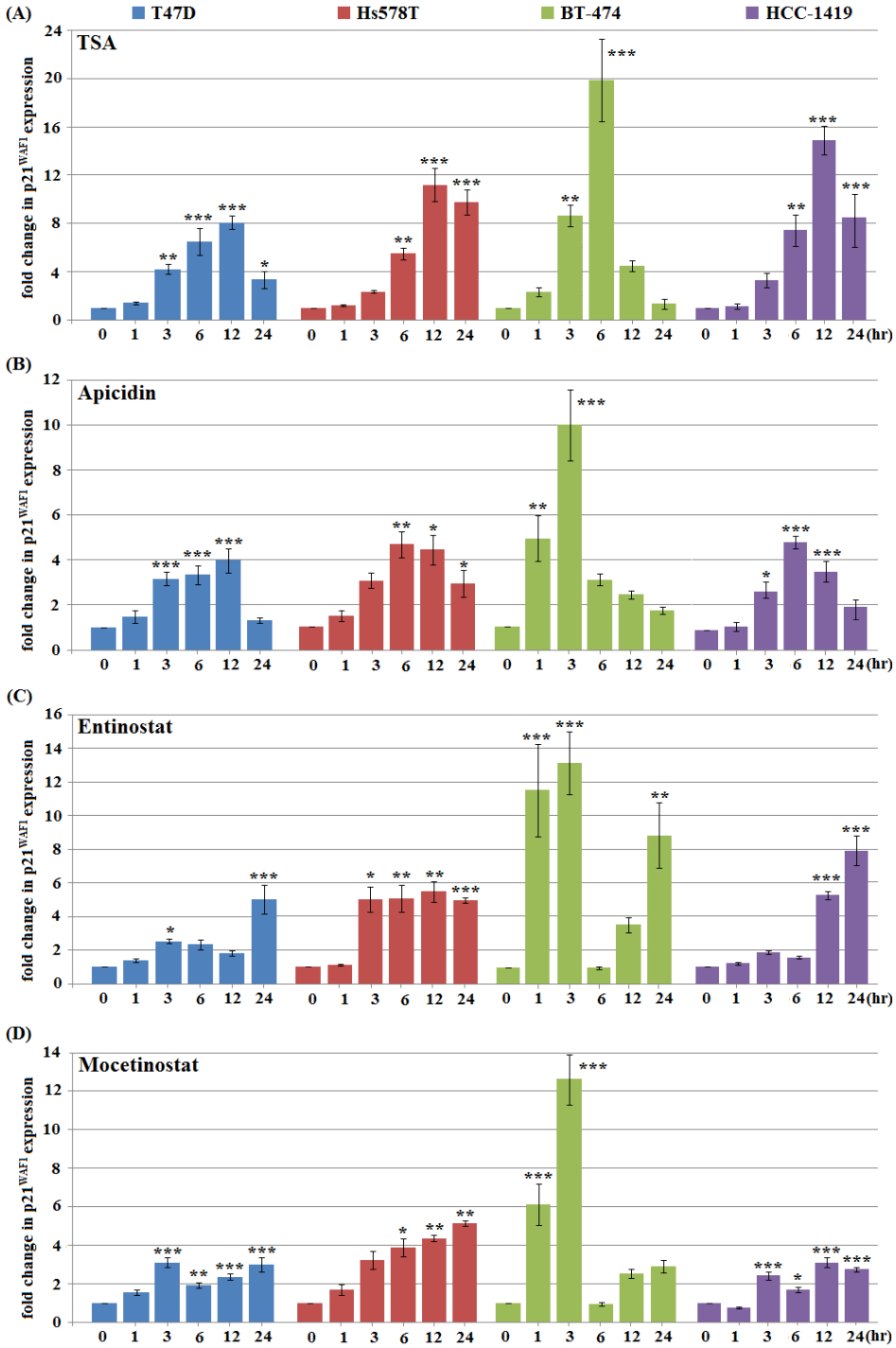

Figure 4.5. $p21^{WAF1}$ mRNA induction following HDI treatment. T47D, Hs578T, BT-474 or HCC-1419 cells were treated with (A) TSA, (B) Apicidin, (C) Entinostat or (D) Mocetinostat and collected in Qiazol Lysis Buffer at the time points indicated. Total RNA was purified and mRNA reverse transcribed, and $p21^{WAF1}$ levels were assessed with gene specific primers using RT-qPCR normalised against RPL13A. Fold changes were calculated by the ΔΔCt method, data represent the mean of three independent experiments done in triplicate, +/- SEM. Asterisks indicate significance at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

μM TSA, 2 μM Apicidin, 2 μM Entinostat and 1 μM Mocetinostat) still induced $p21^{WAF1}$ expression, albeit to a lesser degree (data not shown).

The BT-474 cell line exhibited rapid $p21^{WAF1}$ induction, with a biphasic response to 2 μM Entinostat, while TSA (1 μM), Apicidin (2 μM) and Mocetinostat (1 μM) transiently increased expression. The maximal mRNA levels were observed at 6 hr in TSA-treated cells (19.93 fold [$p<0.001$]), 3 hr in Apicidin-treated cells (10.00 fold [$p<0.001$]), 3 hr in Entinostat-treated cells (13.14 fold [$p<0.001$]) and 3 hr in Mocetinostat treated cells (12.65 fold [$p<0.001$]). Despite enhanced histone acetylation, these compounds only transiently induced $p21^{WAF1}$ in this cell line.

The expression of $p21^{WAF1}$ in the HCC-1419 cell line increased following HDI treatment; 1 μM TSA and 2 μM Apicidin-mediated induction was transient whereas 2 μM Entinostat and 1 μM Mocetinostat had longer effects. The induction following TSA treatment was 14.87 fold ($p<0.001$) at 12 hr, 4.87 fold ($p<0.001$) at 6 hr in Apicidin treated cells, and 7.91 fold ($p<0.001$) and 3.01 fold ($p<0.001$) in Entinostat- and Mocetinostat-treated cells, respectively.

In general, the effects following the class I-specific HDI treatments were less striking than those observed with the 'pan-specific' TSA. With the exception of the BT-474 cell line, the benzamides sustained $p21^{WAF1}$ induction over 24 hr; in addition, this cell line had the maximum $p21^{WAF1}$ induction following HDI treatment. Furthermore, Entinostat and Mocetinostat exhibited a biphasic pattern unique to these benzamides in the BT-474 cell line.

4.1.4 Summary

The data indicate these HDIs are anti-proliferative, lead to acetylated histones and induce $p21^{WAF1}$ expression in the breast cancer cell lines; however, several differences can be highlighted between them. While these compounds similarly enhanced the acetylation of histones in a dose-dependent and time-dependent manner in each the four cell lines, Entinostat and Mocetinostat treatment required a higher concentration to decrease cell viability by 50%. In fact, with the exception of TSA treatment in the Hs578T cells, the cell lines required higher dosages of the benzamides to induce cytotoxicity. In addition, the cell viability profiles from Apicidin-treated cells were similar between the cell lines. $p21^{WAF1}$ induction was rapid and transient in the BT-474 cell line; in addition, the effect of Entinostat-mediated up-regulation was biphasic. TSA showed the maximal induction of $p21^{WAF1}$ in the four cell lines, while the benzamides had moderate effects in the T47D and HCC-1419 cells.

4.2 The Response of SRC and MYC to Histone Deacetylase Inhibitor Treatment

It has been previously established that SRC gene expression is repressed following TSA and NaB treatment in many cancer-derived cell lines (Kostyniuk *et al*., 2002). This transcriptional repression has also been observed for the proto-oncogene MYC (Wang *et al*., 1998; Sasakawa *et al*., 2003; Xu *et al*. 2005; Ierano *et al*., 2013, Leone *et al*., 2015; Raha *et al*., 2015; Bonham and Beaton-Brown, unpublished data). In addition, class- and isoform-specific HDIs have been shown to exhibit differential effects on gene expression (Glaser *et al*., 2003; Halsall *et al*., 2012; Chatterjee *et al*., 2013). It has been demonstrated that HDI-mediated repression is not a universal phenomenon. For example, benzamide treatment had no effect on SRC and MYC expression (Bonham and Beaton-Brown, unpublished data). The extent of HDI-mediated repression of these proto-oncogenes in various cancer-derived cell lines is unclear. Therefore, SRC and MYC expression were analysed following HDI treatment, in order to examine the characteristics of this transcriptional repression.

4.2.1 Relative Steady-State Levels of SRC and MYC

To better assess the response of SRC and MYC transcription to HDI treatment, the relative steady-states of transcripts for these proto-oncogenes were assessed in the breast cancer cell lines (Figure 4.6). The data was normalised to the BT-474 cell lines and the relative level of transcript of SRC and MYC determined between these four cell lines. The T47D cells exhibited high SRC mRNA levels and moderate MYC expression relative to the other three cell lines. The reciprocal was observed in the Hs578T cell line; MYC levels were highest and SRC levels were intermediate. The BT-474 cells exhibited minimal SRC and MYC relative to the other cell lines, while the HCC-1419 cell line had moderate MYC and low SRC transcript levels.

4.2.2 The Expression of SRC Differed in the Breast Cancer Cell Lines

The ability of the compounds to repress SRC at the transcript level differed between the four breast cancer cell lines (Figure 4.7). SRC transcripts in the T47D cell line were repressed

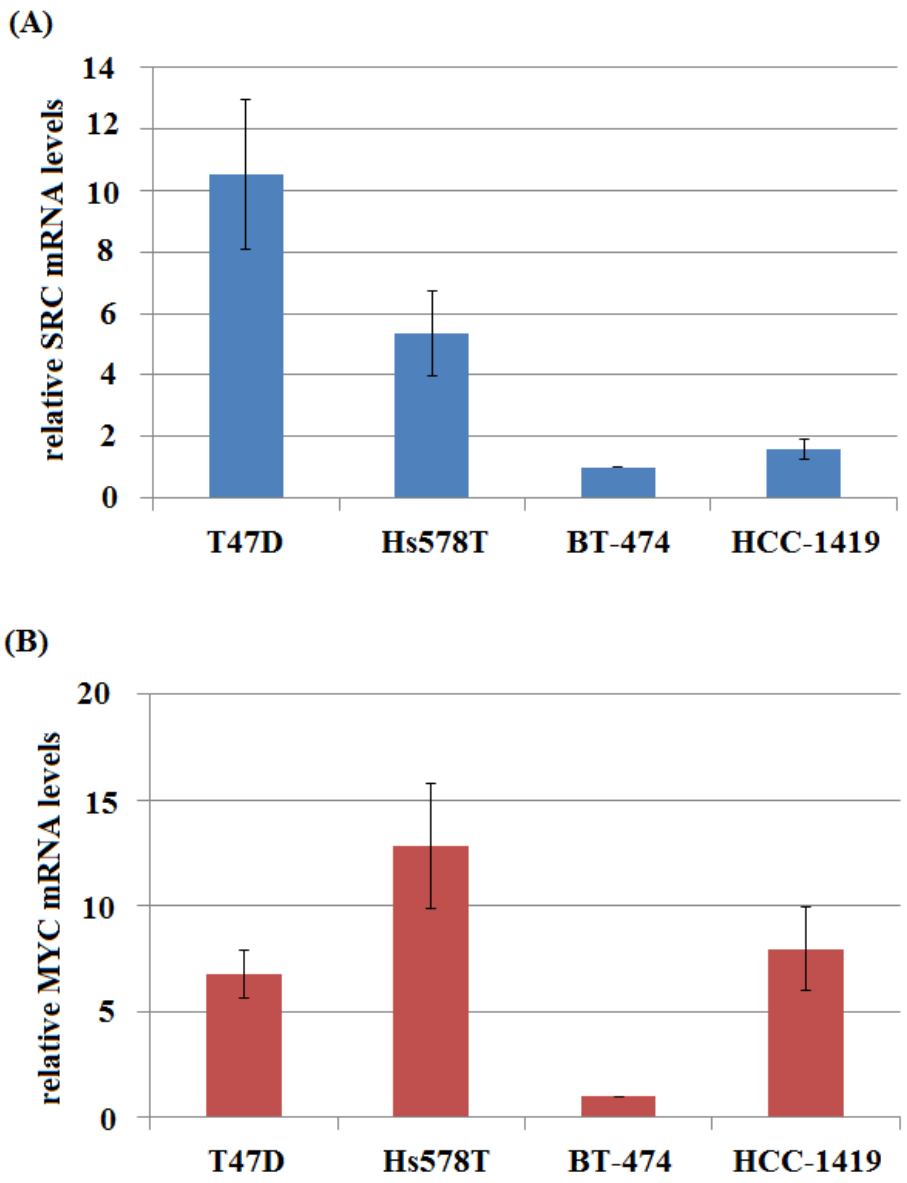

Figure 4.6. Relative SRC and MYC mRNA levels in T47D, Hs578T, BT-474 and HCC-1419 breast cancer cell lines. Total RNA was harvested in Qiazol Lysis Buffer and cDNA synthesized from purified RNA. (A) SRC and (B) MYC levels were assessed with gene-specific primers using RT-qPCR normalised against RPL13A. The relative expressions were calculated by the ΔΔCt method relative to the BT-474 cell line, and data represent the mean of three independent experiments done in triplicate, +/- SEM.

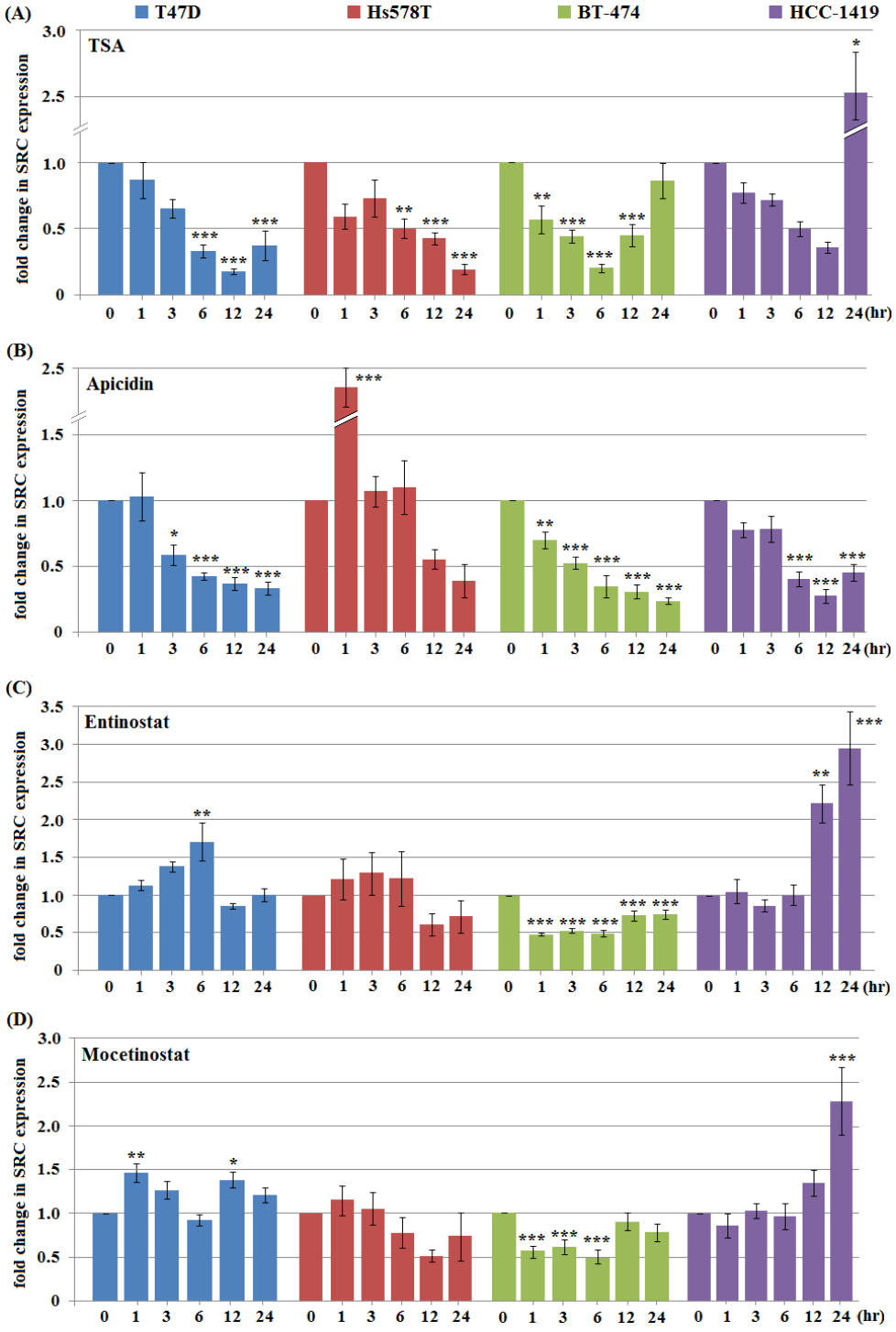

Figure 4.7. Differential SRC expression upon HDI treatment. T47D, Hs578T, BT-474 or HCC-1419 cells were treated with (A) 1 µM TSA, (B) 2 µM Apicidin, (C) 2 µM Entinostat or (D) 1 µM Mocetinostat at the indicated time points and collected in Qiazol Lysis Buffer. Total RNA was purified and reverse transcribed. SRC levels were assessed with SRC-specific primers using RT-qPCR normalised against RPL13A. The fold changes were calculated by the ΔΔCt method, and data represent the mean of at least two independent experiments done in triplicate, +/- SEM. Asterisks indicate significance at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

with 1 µM TSA and 2 µM Apicidin treatment, whereas 2 µM Entinostat and 1 µM Mocetinostat had no effect. While Apicidin treatment sustains SRC repression after 24 hr treatment at 0.33 fold ($p<0.001$), TSA exhibits transient properties; the maximal repression is 0.18 fold ($p<0.001$) at 12 hr. Comparable results were observed in the Hs578T cell line. Both 2 µM TSA and 5 µM Apicidin maintained SRC repression after 24 hr treatment (0.19 fold [$p<0.001$] and 0.39 fold [$p<0.001$]). While 5 µM benzamide treatment did not result in statistical significance, repression is observed at following 12 hr treatment. It is interesting to note that lower concentrations of TSA and Apicidin (1 µM and 2 µM, respectively) down-regulated SRC expression, although not to the extent observed with the higher doses (data not shown). In addition, a lower concentration of the benzamides (2 µM Entinostat and 1 µM Mocetinostat) had no effect on SRC mRNA in the Hs578T cell line (data not shown).

In the BT-474 cell line, TSA and Apicidin repressed SRC expression, and Apicidin repressed SRC to a greater extent than TSA. Modest SRC repression was also observed after Entinostat and Mocetinostat treatment. The effects of TSA and the benzamides in the BT-474 cell line were transient; maximal down-regulation was observed at 6 hr treatment (0.20 fold [$p<0.001$]) in TSA-treated cells, and at 6 hr treatment (0.49 fold [$p<0.001$] and 0.51 fold [$p<0.001$]) in both Entinostat- and Mocetinostat-treated cells. The effects of Apicidin were not transient and repression maintained after 24 hr treatment (0.24 fold [$p<0.001$]).

SRC expression in the HCC-1419 cells was increased with Entinostat and Mocetinostat, and repressed with TSA and Apicidin. However, while repression was initially observed 6 hr and 12 hr (0.36 fold) after TSA treatment, expression rebounded near basal levels at 2.5 fold ($p<0.05$) at 24 hr treatment. Treatment with Apicidin maintained repression for an extended period of time (0.28 fold [$p<0.001$]), however levels began to rebound at 24 hr. Interestingly, Entinostat and Mocetinostat treatment induced SRC mRNA, with a 2.9 fold ($p<0.001$) and a 2.4 fold ($p<0.001$) increase at 24 hr.

4.2.3 The Expression of MYC Differed in the Breast Cancer Cell Lines

The breast cancer cell lines exhibited differential responses of MYC after HDI treatment (Figure 4.8). Following TSA and Apicidin treatment in the T47D cells, the levels of MYC were repressed. While the effects of Apicidin were not as striking (0.56 fold [$p<0.05$]) and were short-

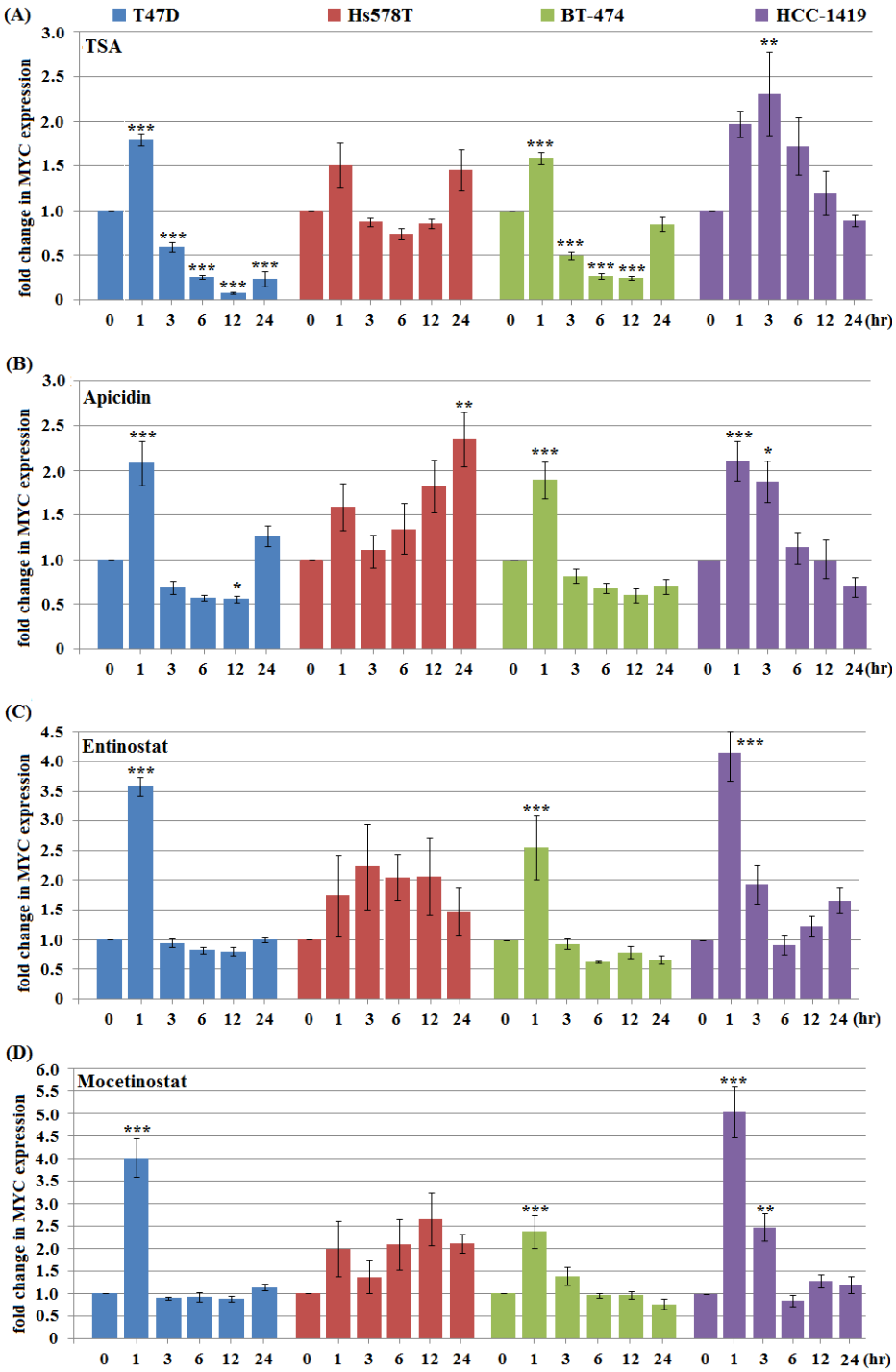

Figure 4.8. Differential MYC expression following HDI treatment. T47D, Hs578T, BT-474 or HCC-1419 cells were treated with (A) 1 µM TSA, (B) 2 µM Apicidin, (C) 2 µM Entinostat or (D) 1 µM Mocetinostat and collected in Qiazol Lysis Buffer at the indicated time points. Total RNA was purified and the mRNA reverse transcribed, and MYC levels were assessed with MYC-specific primers using RT-qPCR normalised against RPL13A. The fold changes were calculated by the ΔΔCt method, and data represent the mean of at least two independent experiments done in triplicate, +/- SEM. Asterisks indicate significance at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

lived, TSA repressed expression 0.08 ($p<0.001$) at 12 hr treatment. The benzamides induced MYC initially at 1 hr and/or 3 hr treatment, but otherwise had little effect.

HDI-mediated effects on MYC expression were absent in the Hs578T cells; in fact, treatment modestly increased expression over 24 hr. In addition, treatment with the lower concentrations of the drugs (1 µM TSA, 2 µM Apicidin, 2 µM Entinostat and 1 µM Mocetinostat) in the Hs578T cell line did not significantly differ from the MYC expression profiles obtained with the higher concentrations (data not shown).

TSA repressed MYC in the BT-474 cell line, whereas Apicidin, Entinostat and Mocetinostat initially induced expression at 1 hr (1.91 fold [$p<0.001$], 2.56 fold [$p<0.001$] and 2.38 fold [$p<0.001$], respectively) without a long-term effect. In addition, the effects of TSA were transient and the maximal repression observed was 0.24 fold ($p<0.0001$) after 12 hr treatment.

The HCC-1419 cell line initially exhibited MYC induction following HDI treatment. This transient induction was observed after 1 hr to 3 hr treatment at 2.31 fold ($p<0.01$), 2.12 fold ($p<0.001$), 4.14 fold ($p<0.001$) and 5.03 fold ($p<0.001$) in TSA, Apicidin, Entinostat and Mocetinostat treated cells, respectively.

4.2.4 Summary

While SRC and MYC are generally down-regulated following TSA and NaB treatment in multiple cancer-derived cell lines (Kostynuik *et al.*, 2002), the data illustrates that the HDI-mediated repression of these proto-oncogenes is not a universal effect in the four breast cancer cell lines tested. SRC repression was confirmed following TSA and Apicidin treatment, with the exception of induction at 24 hr treatment in the HCC-1419 cell line. Likewise, it was confirmed that the benzamides either had no effect or induced expression in three cell lines (T47D, Hs578T and HCC-1419), but only repressed SRC in the BT-474 cells.

The response of MYC did not mimic that observed with SRC expression following HDI treatment. While the benzamides either induced or had no effect on MYC expression in all these cell lines, TSA and/or Apicidin only repressed expression in the T47D and BT-474 cell lines. Furthermore, the Hs578T cell line exhibited the least response to HDI-mediated MYC induction or repression; this could reflect minimal histone acetylation following class I-specific treatment

or the increased steady-state MYC levels. In addition, the different subtypes of breast cancer cells respond differently to HDI treatment. The MYC locus is amplified in the triple-negative Hs578T and HER2-amplified HCC-1419 cell lines (Kao *et al*., 2009), and the data indicate that these two subclasses could not clinically benefit from HDI treatment, as the ERα-positive cell lines (T47D and BT-474).

4.3 HDI Treatment Induces miRNA Gene Expression

It has been extensively demonstrated in the literature that oncogenic and/or tumour suppressive miRNA are altered upon HDI treatment (Scott *et al.,* 2006; Lu *et al.,* 2005; Tsai *et al*., 2011; Saito *et al*., 2006). For example, miR-409 and miR-127 were up-regulated following HDI treatment in cancer-derived cell lines (Tsai *et al*., 2011; Saito *et al*., 2006). It has also been demonstrated that miRNA are subjected to differential effects following class- and/or isoform-specific drug administration; in fact, it has been established that Entinostat induced expression of miR-125a and miR-126b in HER2-enriched cell lines, whereas hydroxamate treatment had no effect (Wang *et al*., 2013). Therefore, it was hypothesised that miRNA could be differentially regulated by HDI treatment in the four breast cancer cell lines. In order to assess this, cell lines were treated with 1 µM TSA, 2 µM Apicidin, 2 µM Entinostat or 1 µM Mocetinostat, and the expression of miRNA implicated in breast cancer progression were analysed by RT-qPCR. It was observed that three miRNA genes, miR-129-5p, miR-424 and miR-9-3p, were induced in the T47D, Hs578T and/or HCC-1419 cell lines upon HDI treatment.

4.3.1 miR-129-5p, miR-424 and miR-9-3p Induction

Differential effects upon miRNA induction were observed in the four breast cancer cell lines following HDI treatment. In the T47D cell line, Entinostat and TSA treatment induced miR-129-5p expression, whereas Apicidin and Mocetinostat has no effect (Figure 4.9). TSA and Entinostat treatment up-regulated miR-129-5p expression 5.72 fold ($p<0.001$) and 4.46 fold ($p<0.01$), respectively. This miRNA was not increased with treatment in the other cell lines.

miR-424 expression was induced over time with TSA and benzamide treatment in both the T47D and Hs578T cell lines, while Apicidin had no effect (Figure 4.10). The benzamides up-

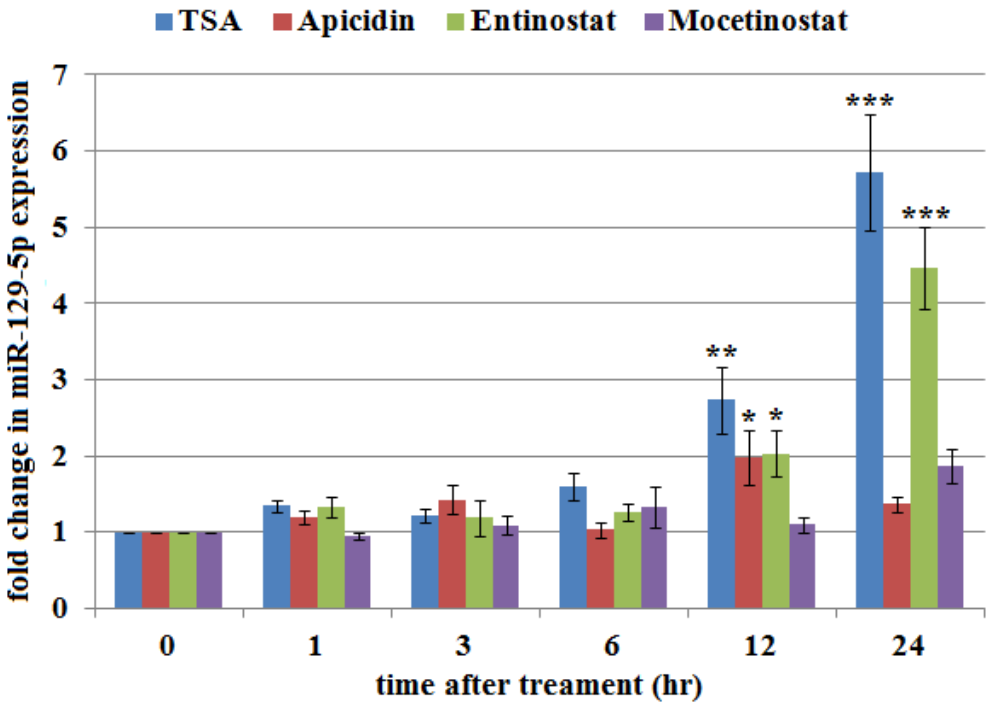

Figure 4.9. miR-129-5p induction following HDI treatment in the T47D cell line. Total RNA from untreated and 1 μM TSA, 2 μM Apicidin, 2 μM Entinostat or 1 μM Mocetinostat treated T47D cells were collected at the indicated time points. Samples were subjected to RT-qPCR with miR-129-5p-specific primer, and the fold changes calculated by the ΔΔCt method. Data shown is the averaged results of three independent experiments with at least three technical repeat(s) +/- SEM, and asterisks indicate significance at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

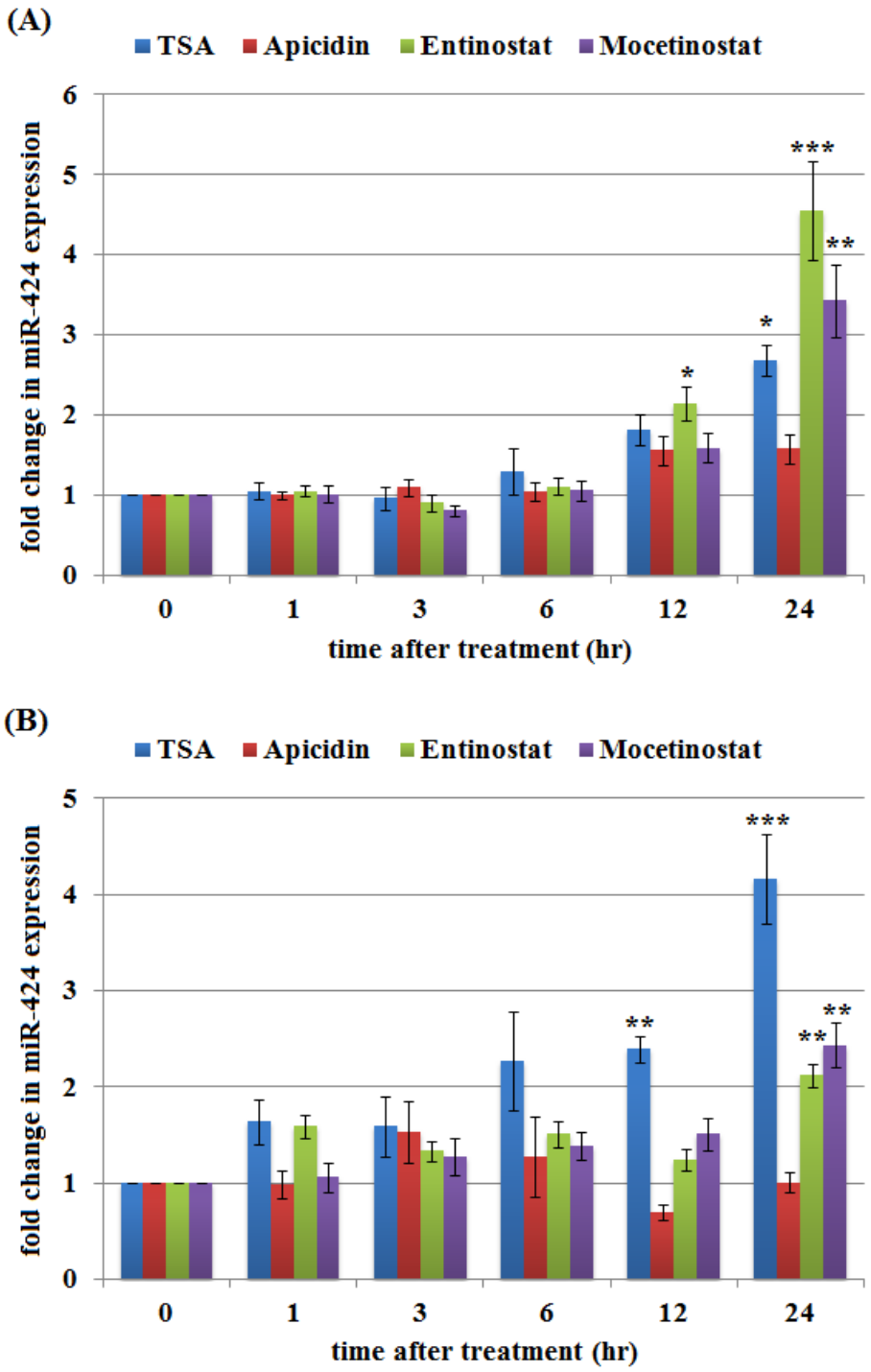

Figure 4.10. miR-424 induction in HDI-treated T47D and Hs578T cell lines. Total RNA from untreated and 1 μM TSA, 2 μM Apicidin, 2 μM Entinostat or 1 μM Mocetinostat treated (A) T47D or (B) Hs578T cells were collected at the indicated time points. Samples were subjected to RT-qPCR with miR-424-specific primers, and the fold changes calculated by the ΔΔCt method. Data shown is the averaged results of three independent experiments with at least three technical repeat(s) +/- SEM, and asterisks indicate significance at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

regulated expression 4.55 fold ($p<0.001$) and 3.42 fold ($p<0.01$) while TSA induced expression 2.69 fold ($p<0.05$) at 24 hr treatment in the T47D cells. In the Hs578T cell line, 24 hr treatment with TSA induced expression 4.16 fold ($p<0.001$), while Entinostat and Mocetinostat up-regulated expression 2.11 fold ($p<0.01$) and 2.43 fold ($p<0.01$), respectively.

HDI treatment induced miR-9-3p expression in the T47D, Hs578T and HCC-1419 cell lines (Figure 4.11). In the T47D cell line, TSA increased expression 20.70 fold ($p<0.001$) at 24 hr treatment, while Apicidin and the benzamides only up-regulated expression 3.88 fold ($p<0.01$), 5.64 fold ($p<0.01$) 3.14 fold ($p<0.01$), respectively. miR-9-3p expression increased 12.94 fold ($p<0.001$) following 24 hr TSA treatment, while Apicidin, Entinostat and Mocetinostat induced expression 2.14 fold ($p<0.05$), 2.65 fold ($p<0.05$) and 2.40 fold ($p<0.05$) in the Hs578T cells. Similarly in the HCC-1419 cell line, TSA induced expression 3.82 fold ($p<0.001$), while the class I-specific HDIs had no effect after 24 hr.

4.3.2 Down-Regulation of miRNA Gene Targets

miRNA-mediated gene regulation involves the decay of mRNA transcripts and/or translational inhibition dependent upon the complementary binding of the seed sequence to the mRNA (Bartel *et al*., 2004; Bagga *et al*., 2005). Thus, utilising the bioinformatic programmes miRanda, TargetScan and Diana, which predict miRNA:mRNA interactions based on computorial algorithms (Enright *et al*., 2003; Betel *et al*., 2008, 2010; Vlachos *et al*., 2014), miRNA target genes with potential oncogenic processes in breast cancer were validated. The binding of miRNA to the 3' UTR of their target mRNA are discussed in more detail in the following sections. These targets were analysed for mRNA and/or protein expression following HDI-mediated miRNA induction and miRNA transfection. It was hypothesised that the biologically significant induction of miRNA down-regulated their gene targets at the mRNA and/or protein expression levels.

In order to eliminate HDI-mediated effects independent of miRNA induction, the cell lines were additionally transfected with synthetic mature miRNA obtained from Qiagen. These are synthetically produced double-stranded RNA molecules that are processed into mature miRNA by the RISC machinery and able to exert effects within the cells. It has been illustrated in the literature that transfecting cells with synthetic miRNA affects target mRNA and/or protein

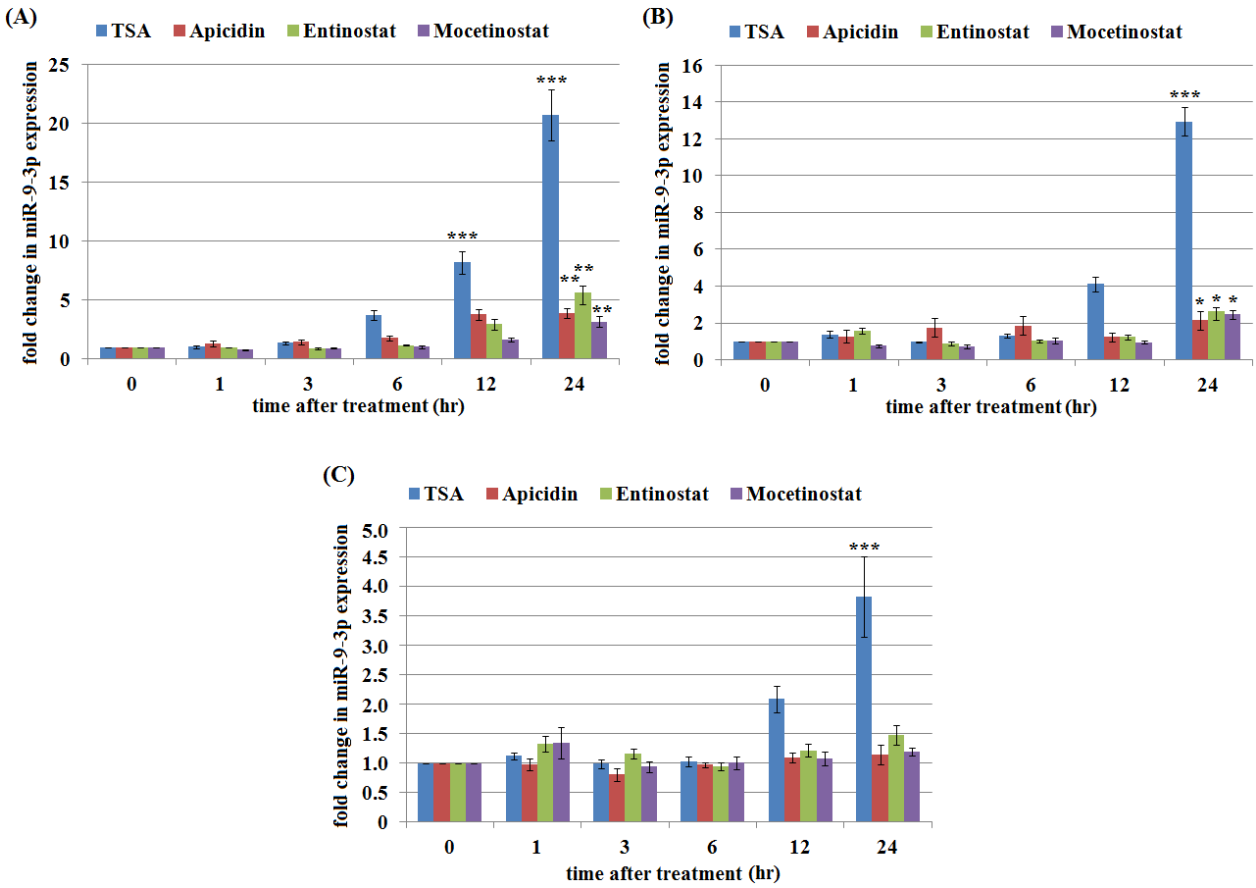

Figure 4.11. miR-9-3p induction upon HDI treatment in T47D, Hs578T and HCC-1419 cell lines. Total RNA from untreated and 1 μM TSA, 2 μM Apicidin, 2 μM Entinostat or 1 μM Mocetinostat treated (A) T47D, (B) Hs578T or (C) HCC-1419 cells were collected at the indicated time points. Samples were subjected to RT-qPCR with miR-9-3p-specific primers, and the fold changes calculated by the ΔΔCt method. Data shown is the averaged results of three technical repeat(s) +/- SEM, and asterisks indicate significance at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

expression (Ghosh *et al.*, 2010; Roccaro *et al.*, 2010). Thus, protein expression of their respective gene targets were subsequently analysed in the transfected cells, to further validate them as potential miRNA targets.

4.3.2.1 The Down-Regulation of ERα, a miR-129-5p Gene Target, in the T47D Cell Line

The miRanda and TargetScan miRNA:mRNA interaction databases indicate that the ERα gene (ESR1) is a potential target for miR-129-5p, which is induced following TSA and Entinostat treatment in the T47D cell line. ERα possesses three potential miR-129-5p binding sequences spaced closely together at the middle region of the 3' UTR (Figure 4.12). Although miR-129-5p had been induced in both the T47D and Hs578T cell lines following HDI treatment, ERα mRNA and protein expression were only examined in the ERα-positive T47D cells.

The transcript levels of ERα were down-regulated -10 fold ($p<0.0001$) over 24 hr with TSA treatment (Figure 4.13), whereas Apicidin and Entinostat were transient in nature and maximally repressed expression -2 fold ($p<0.001$) after 12 hr and -2 fold ($p<0.001$) after 6 hr treatment, respectively. Mocetinostat did not affect ERα expression. However, this repression did not reflect the induction of miR-129-5p in the T47D cell line; while TSA and Entinostat treatment resulted in the highest miR-129-5p induction, only TSA significantly reduced ERα transcript levels.

In addition, the protein expression of ERα reflected the mRNA data; TSA treatment down-regulated ERα (Figure 4.14), whereas expression was not altered with Apicidin and benzamide treatment (data not shown). It has previously been demonstrated in the literature that treatment with 'pan-specific' HDIs such as TSA and Vorinostat represses ERα expression, by mechanisms which include enhanced acetylation of the protein and subsequent degradation (Fiskus *et al.*, 2007; Kim *et al.*, 2010). Thus, it is possible that HDI-mediated mechanisms independent to or in addition to miR-129-5p induction are responsible for the repression.

To test this, miR-129-5p was transfected into T47D cells, and the protein expression of ERα determined. Following a 24 hr transfection, immuno-blot analysis indicated that the protein levels of ERα were unaltered (data not shown). Therefore, it is likely that the HDI-mediated repression of ERα is due to varying downstream effects of the drugs, rather than induction of miR-129-5p, in this cell line.

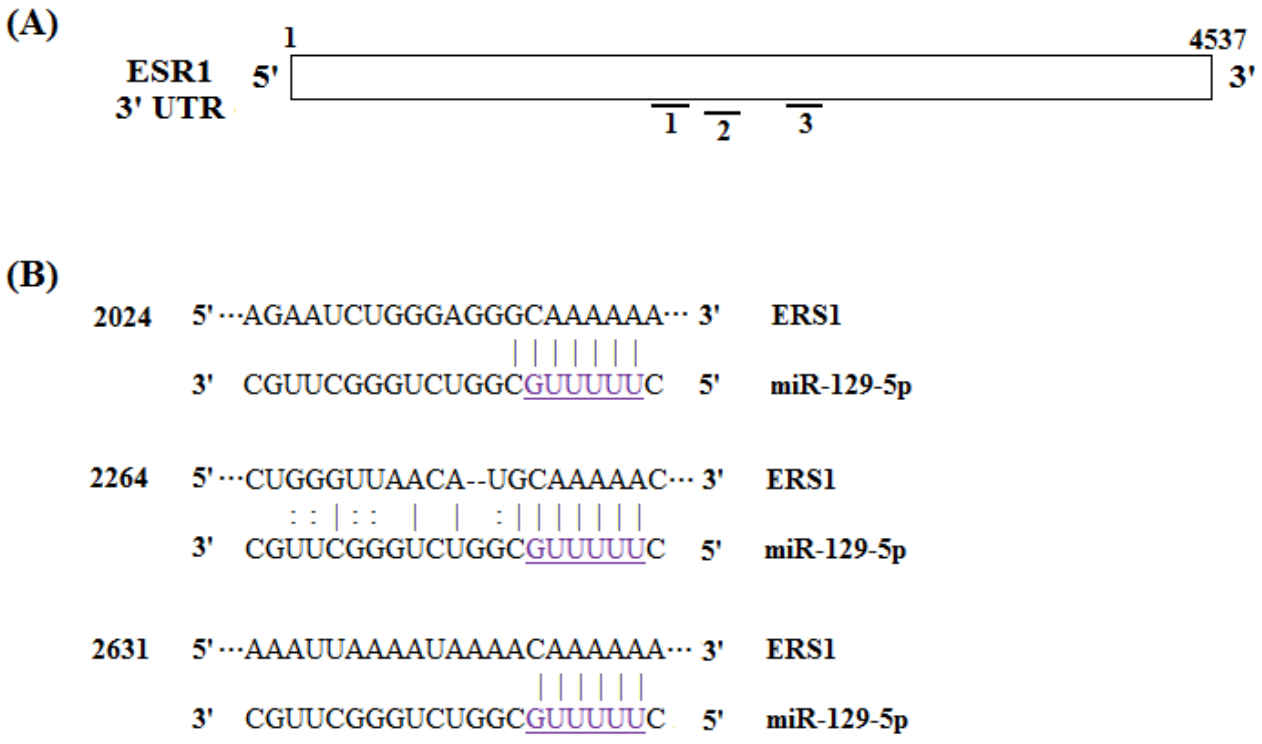

Figure 4.12. miR-129-5p binding sites in the 3' UTR of the ESR1 gene. A schematic representation of the (A) human ESR1 3' UTR with miR-129-5p binding sites. The nucleotide sequence numbers within the 3' UTR are labelled above. (B) The nucleotide duplex formation between the miRNA and mRNA. The vertical lines indicate predicted base pairing and colons indicate wobble base pairing. The seed sequence of miR-129-5p is underlined in purple font.

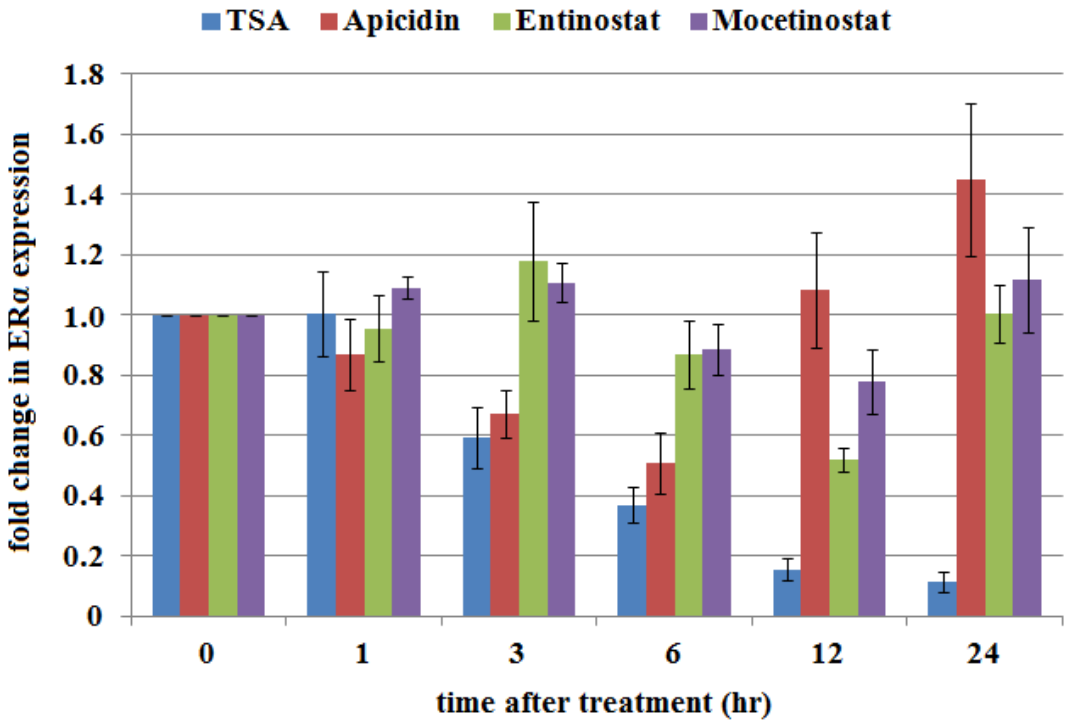

Figure 4.13. ERα expression following HDI treatment. T47D cells were treated with 1 µM TSA, 2 µM Apicidin, 2 µM Entinostat or 1 µM Mocetinostat and collected in Qiazol Lysis Buffer at the indicated times. Untreated and drug vehicle controls were also harvested at this time. Total RNA was purified and the mRNA reverse transcribed, and ERα levels were assessed with ERα-specific primers using RT-qPCR normalised against an internal control. The fold changes were calculated by the ΔΔCt method, and data represent the mean of one independent experiment done in triplicate. The error bars represent the standard deviation between the triplicates.

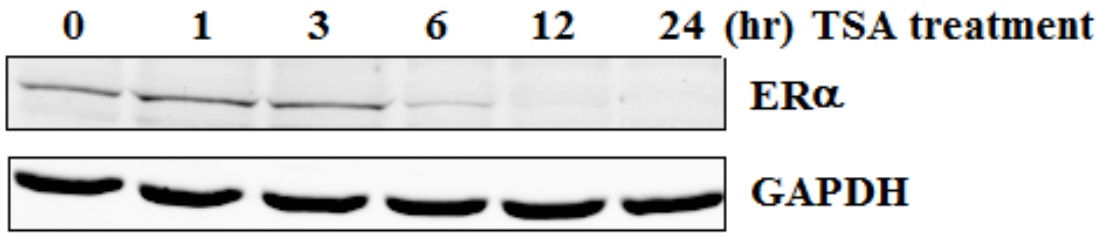

Figure 4.14. ERα expression in TSA-treated T47D cells. The cells were treated with 1 μM TSA over a period of 24 hr and collected in 2X Laemmli sample buffer at the indicated time points. Following SDS-PAGE electrophoresis, 25 μg protein was transferred to a nitrocellulose membrane and incubated with an anti- Rα antibody. GAPDH is included as a loading control. The data shown is representative of two biological repeats, which gave similar results.

4.3.2.2 Chek1, a miR-424 and miR-129-5p Target, is Repressed in T47D and Hs578T Cells

It has recently been demonstrated that miR-424 targets the 3' UTR of Chek1 to decrease protein levels in cervical cancer cell lines (Xu *et al*., 2012). In addition, miRanda and TarBase miRNA:mRNA interaction databases (Enright *et al*., 2003; Betel *et al*., 2008, 2010; Vlachos *et al*., 2014) predict that Chek1 mRNA is a target for both miR-424 and miR-129-5p. It possesses one potential binding site for each miRNA in the 3' UTR (Figure 4.15); the miR-424 site is located near the beginning of the 3' UTR while the miR-129-5p site is located two-thirds from the proximal end.

To validate Chek1 as a potential miR-424 and/or miR-129-5p target in these cell lines, HDI-treated and miRNA-transfected cells were examined for mRNA and protein expression. While no effect on CHEK1 mRNA stability was observed, protein levels were down-regulated following TSA and/or Apicidin in the T47D (Figure 4.16 A) and Hs578T (Figure 4.16 B) cell lines. However, these patterns do not reflect the HDI-mediated induction of miR-129-5p and miR-424 in the cell lines; Apicidin repressed Chek1 protein in the T47D cell line, but had no effect on the expression of either miR-129-5p or miR-424. In addition, Entinostat induced miR-424 4.5 fold ($p<0.001$), but treatment did not affect Chek1 protein levels (data not shown).

Therefore, it was unclear whether protein down-regulation was due to the presence of miRNA within the cell; the biological effects of the HDIs could be responsible. In order to test this, synthetic miR-424 was transfected into the T47D and Hs578T cell lines. Transfection of miR-424 down-regulated Chek1 protein expression over 24 hr in the T47D cell line (Figure 4.17). Although Chek1 contained a potential seed sequence in the 3' UTR, miR-129-5p transfection had no effect on Chek1 protein levels. This indicates that miR-424, but not miR-129-5p, can bind to the 3' UTR of Chek1 mRNA to inhibit translation in the T47D cell line. It is possible that both miR-424 and certain HDIs contribute to the regulation of Chek1.

4.3.2.3 c-MYB, a miR-424 Target, is Repressed by HDIs in T47D and Hs578T Cell Lines

c-MYB is the cellular homologue of the avian myeloblastosis viral oncogene and is a potential target for miR-424. The c-MYB gene encodes a proto-oncogenic transcription factor frequently over-expressed in breast cancer, particularly the ERα-positive and HER2-amplified

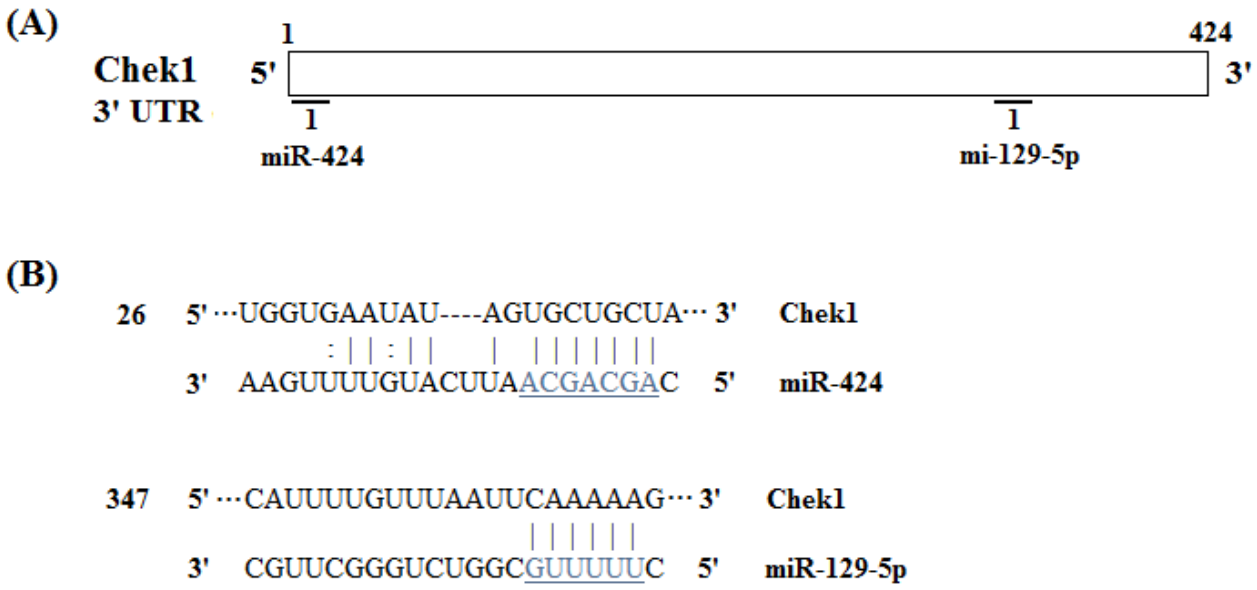

Figure 4.15. miR-129-5p and miR-424 binding sites in the 3' UTR of the Chek1 gene. A schematic representation of the (A) human Chek1 3'UTR with indicated miR-129-5p and miR-424 binding sites. The nucleotide sequence numbers within the 3' UTR are labelled above. (B) The nucleotide duplex formation between the miRNA and mRNA. The vertical lines indicate predicted base pairing and colons indicate wobble base pairing. The seed sequence of miR-129-5p and miR-424 are underlined and labelled in the purple font.

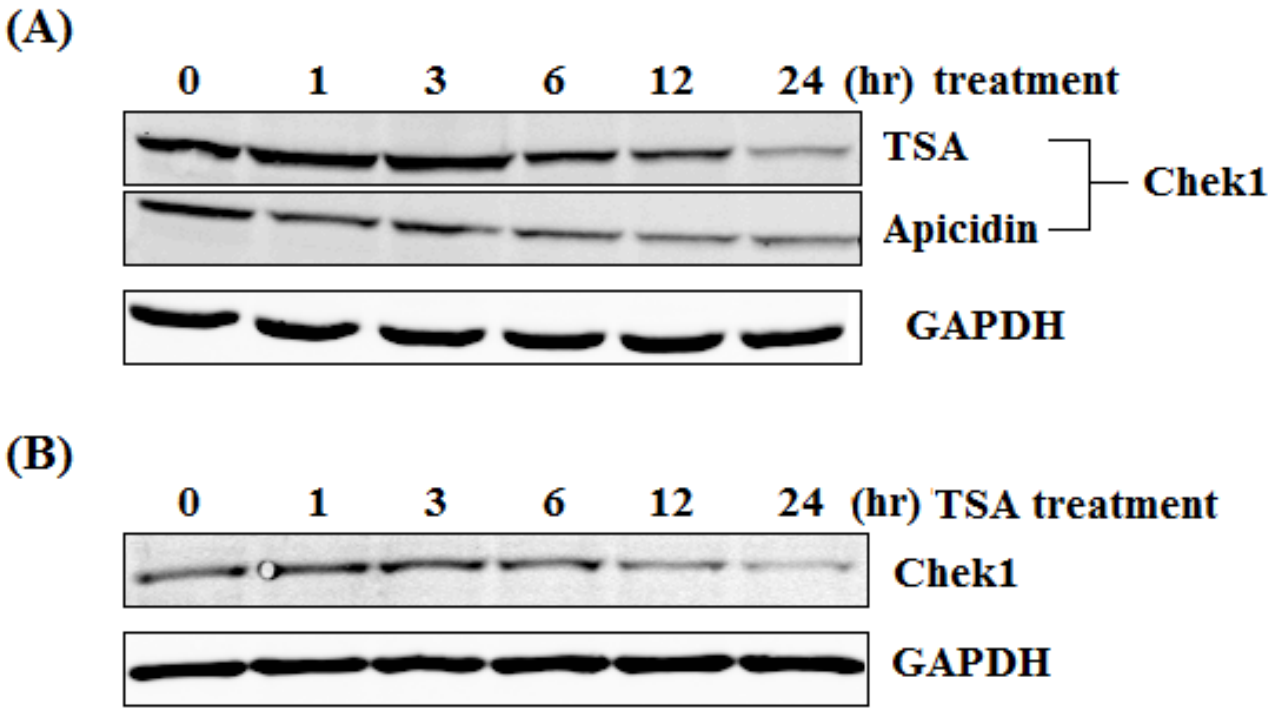

Figure 4.16. Chek1 protein expression following HDI treatment. (A) T47D or (B) Hs578T cells were treated with 1 µM TSA or 2 µM Apicidin over the indicated time points. Cells were harvested in 2X Laemmli sample buffer and 25 µg protein was resolved via SDS-PAGE. Immuno-blotting against Chek1 and GAPDH as a loading control was performed. The data shown is representative of two biological repeats, which gave similar results.

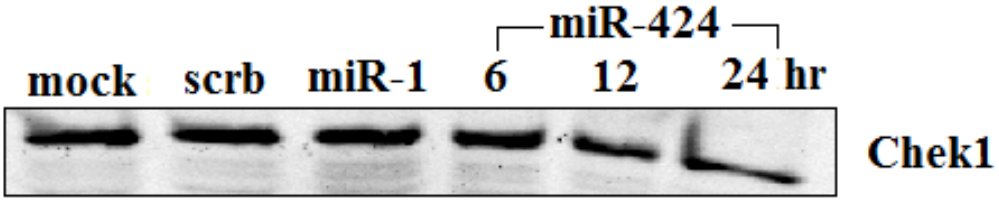

Figure 4.17. miR-424 transfection repressed Chek1 levels in T47D cells. The cells were seeded at 2 x 10^4 cells in 12-well plates and incubated with 300 nM synthetic miR-424 and Lipofectamine 2000 for 4 hr. RPMI-1640 media containing 20% FBS was added to the cells, and they were harvested over 24 hr in 2X Laemmli sample buffer. 25 µg of protein was resolved on a SDS-PAGE gel and immuno-blotting against Chek1 was performed. A mock control containing only Lipofectamine (mock), a scrambled control (scrb) and a non-specific miR-1 control were also included.

subtypes. It is regulated in part by ERα, and therefore c-MYB plays a crucial role in the proliferation of these ERα-positive breast cancer cells and has the ability to repress differentiation and apoptosis (Miao *et al*., 2011). Due to its contributory role in the cellular growth of cancer cells, the expression of c-MYB mRNA and protein levels following HDI-mediated induction of miR-424 were investigated. It was hypothesised that the up-regulation of miR-424 translated to c-Myb protein and/or c-MYB mRNA repression.

c-MYB has two potential, conserved binding sequences for miR-424 in the 3' UTR (Figure 4.18) (Enright *et al*., 2003; Betel *et al*., 2008, 2010; Vlachos *et al*., 2014). The seed sequence binds to two adjacent locations in the 3' UTR of the mRNA. c-MYB expression was down-regulated following HDI-treatment in both the T47D and Hs578T cells (Figure 4.19). In the T47D cell line, c-MYB was repressed -5.71 fold [$p<0.0001$] with TSA and -3.3 fold [$p<0.001$] with Apicidin, while Entinostat and Mocetinostat had no effect. However, in the Hs578T cells, benzamide-mediated repression (-2.5 fold [$p<0.001$]) of c-MYB was comparable to -5 fold ($p<0.0001$) and -2.5 fold ($p<0.001$) repression following TSA and Apicidin, respectively. Immuno-blot analysis of c-Myb protein in HDI-treated or miR-424 transfected cells were inconclusive.

It should be noted that the repression of c-MYB levels did not correlate with the observed induction pattern of miR-424 in either cell line. Apicidin treatment down-regulated c-MYB expression in both cell lines, despite exhibiting no effect on miR-424 induction. In addition, Entinostat and Mocetinostat treatment induced miR-424 to a greater extent than TSA in the T47D cell line, but TSA maximally repressed c-MYB levels whereas the benzamides had only modest effects.

4.3.2.4 Cyclin D, a miR-424 Target, is Repressed Following miR-424 Induction

CCND1 and its protein product cyclin D are frequently over-expressed in many cancers, including breast cancer, and controls the G1/S transition of the cell cycle via binding to CDK4/6 (Grillo *et al*., 2006; Liu *et al*., 2008). It has been demonstrated with luciferase assays that the 3' UTR of CCND1 can be regulated post-transcriptionally by the miR-16 family members, which includes miR-424 (Liu *et al*., 2008). The 3' UTR of CCND1 contains one miR-424 binding site (Figure 4.20). To determine whether the HDI-mediated up-regulation of miR-424 repressed

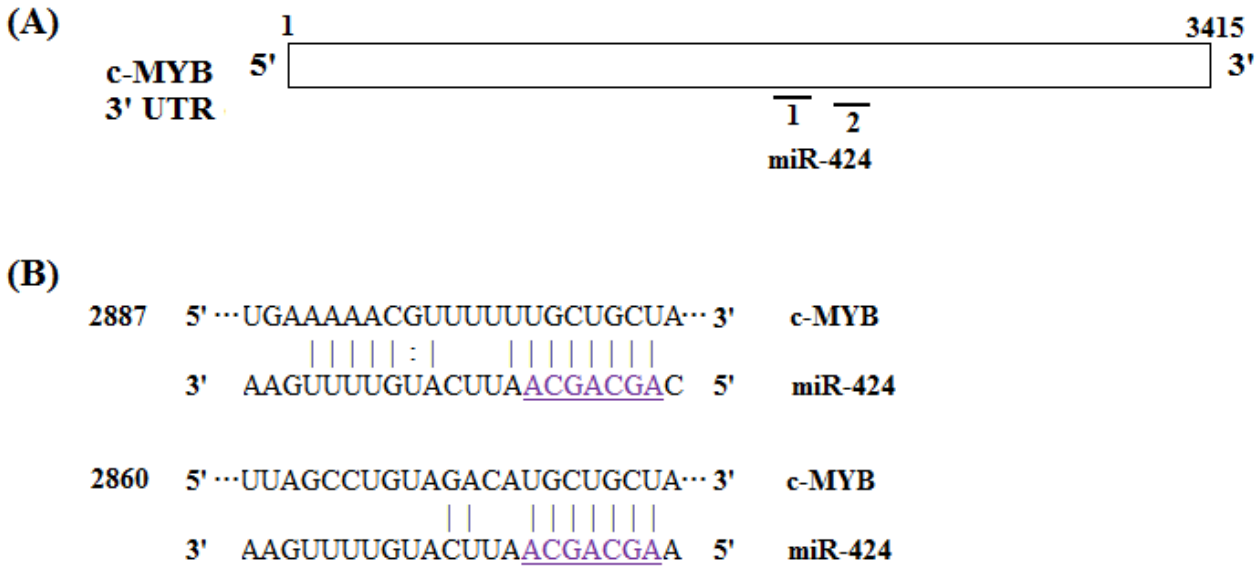

Figure 4.18. miR-424 binding sites in the 3' UTR of the c-MYB gene. A schematic representation of the (A) human c-MYB 3'UTR with indicated miR-424 binding sites. The nucleotide sequence numbers within the 3' UTR are labelled above. (B) The nucleotide duplex formation between the miRNA and mRNA. The vertical lines indicate predicted base pairing and colons indicate wobble base pairing. The seed sequence of miR-424 is underlined in purple font.

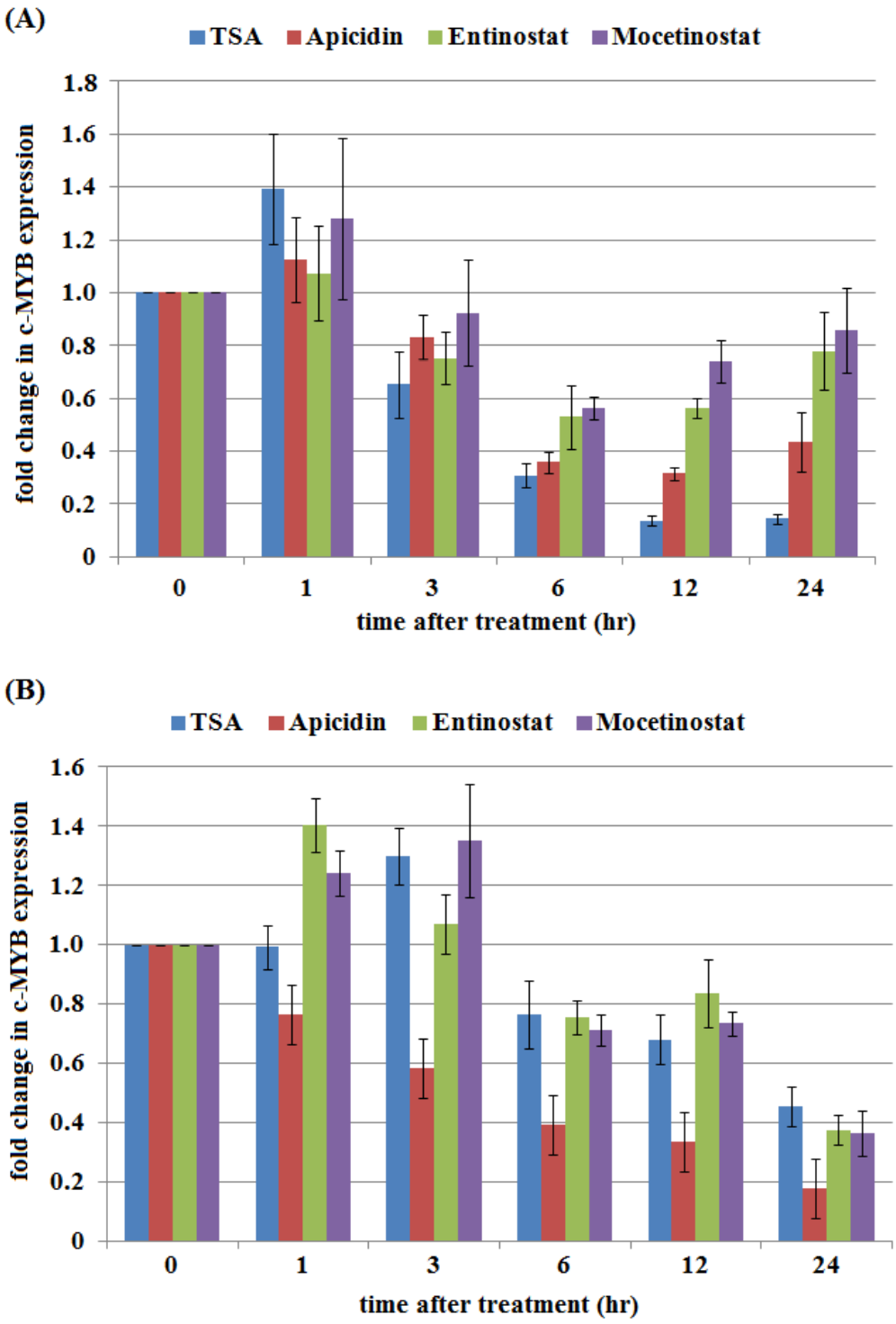

Figure 4.19. c-MYB repression in HDI-treated T47D and Hs578T cells. Total RNA from untreated and 1 μM TSA, 2 μM Apicidin, 2 μM Entinostat or 1 μM Mocetinostat treated T47D cells were collected at the indicated time points. Samples were subjected to Real Time PCR with gene-specific primers, and the fold changes calculated by the ΔΔCt method. Data shown is the averaged results of one technical repeat done in triplicate. The error bars represent the standard deviation between the triplicates.

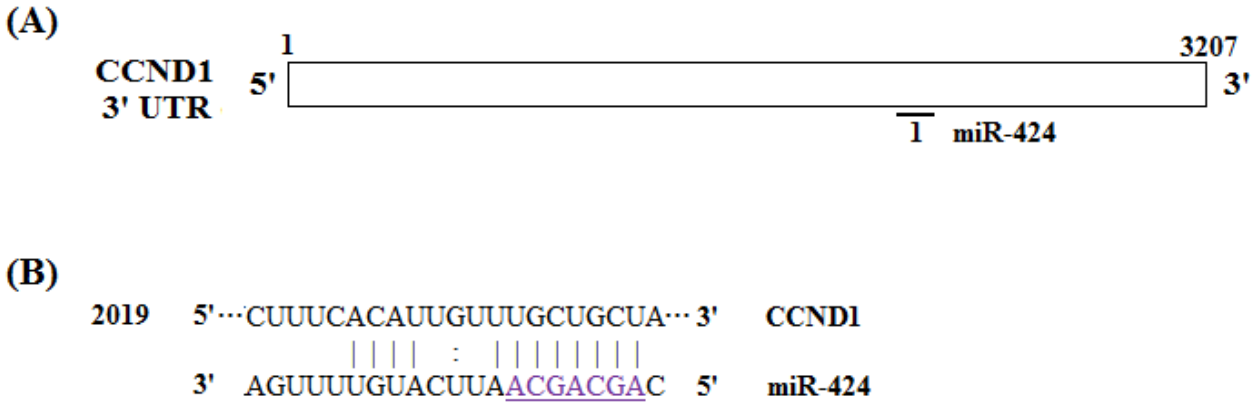

Figure 4.20. miR-424 binding sites in the 3' UTR of the CCNDI gene. A schematic representation of the (A) human CCND1 3'UTR with the indicated miR-424 binding site. The nucleotide sequence numbers within the 3' UTR are labelled above. (B) The nucleotide duplex formation between the miRNA and mRNA (B). The vertical lines indicate predicted base pairing and colons indicate wobble base pairing. The seed sequence of miR-424 is underlined in purple font.

CCND1 translation and/or led to mRNA decay, the protein expression and transcript levels were assessed in the two cell lines.

While the mRNA levels of CCND1 decreased in the T47D cell line (Figure 4.21) following HDI treatment, this repression did not correlate with the HDI-mediated induction of miR-424; Apicidin had no effect on miR-424 induction, although treatment with the cyclic peptide down-regulated CCND1 expression. TSA and the class I-specific Apicidin sustained repression (-10 fold [$p<0.0001$] and -3.3 fold [$p<0.001$]) over 24 hr, whereas the benzamides exhibited modest repression, -2.5 fold ($p<0.001$) and -2.5 fold ($p<0.001$) after 24 hr treatment. Immuno-blot analysis of HDI-treated cells indicated that cyclin D protein levels were down-regulated only following TSA treatment (Figure 4.22). While these protein levels reflect the mRNA repression observed with TSA treatment, discrepancies were observed following treatment with the class I-specific drugs. The protein levels were not significantly altered with Apicidin, Entinostat and Mocetinostat treatments (data not shown), despite mRNA down-regulation.

However, it has been previously reported in the literature that CCND1 transcription is also decreased with HDI treatment (Jin *et al*., 2012; Zhang *et al*., 2012) as well as by miR-424 (Liu *et al*., 2008). It cannot be concluded that the decrease is solely due to increased miR-424 expression, rather than other contributing mechanisms. Therefore, T47D cells were transfected with exogenous miR-424. It was observed that the protein levels of cyclin D were repressed 24 hr after transfection (Figure 4.23), suggesting that miR-424 targets the 3' UTR to inhibit protein synthesis and/or initiate mRNA decay in this cell line.

4.3.2.5 HDAC5, a miR-9-3p Target, is Down-Regulated Following HDI treatment

It has been implicated in the literature that miR-9-3p has a role in acetylation and deacetylation processes, and represses the class IIa HDACs 4 and 5. In B-cell lymphoma lines, up-regulation of miR-9-3p leads to a reduction of these HDACs and increased acetylation (Roccaro *et al*., 2010). Therefore, the mRNA and protein expression of these HDACs were analysed following HDI-mediated up-regulation of miR-9-3p.

The mRNA levels of these enzymes were induced or not significantly altered within 24 hr of HDI treatment in the three cell lines (data not shown). However, HDAC5 protein levels were

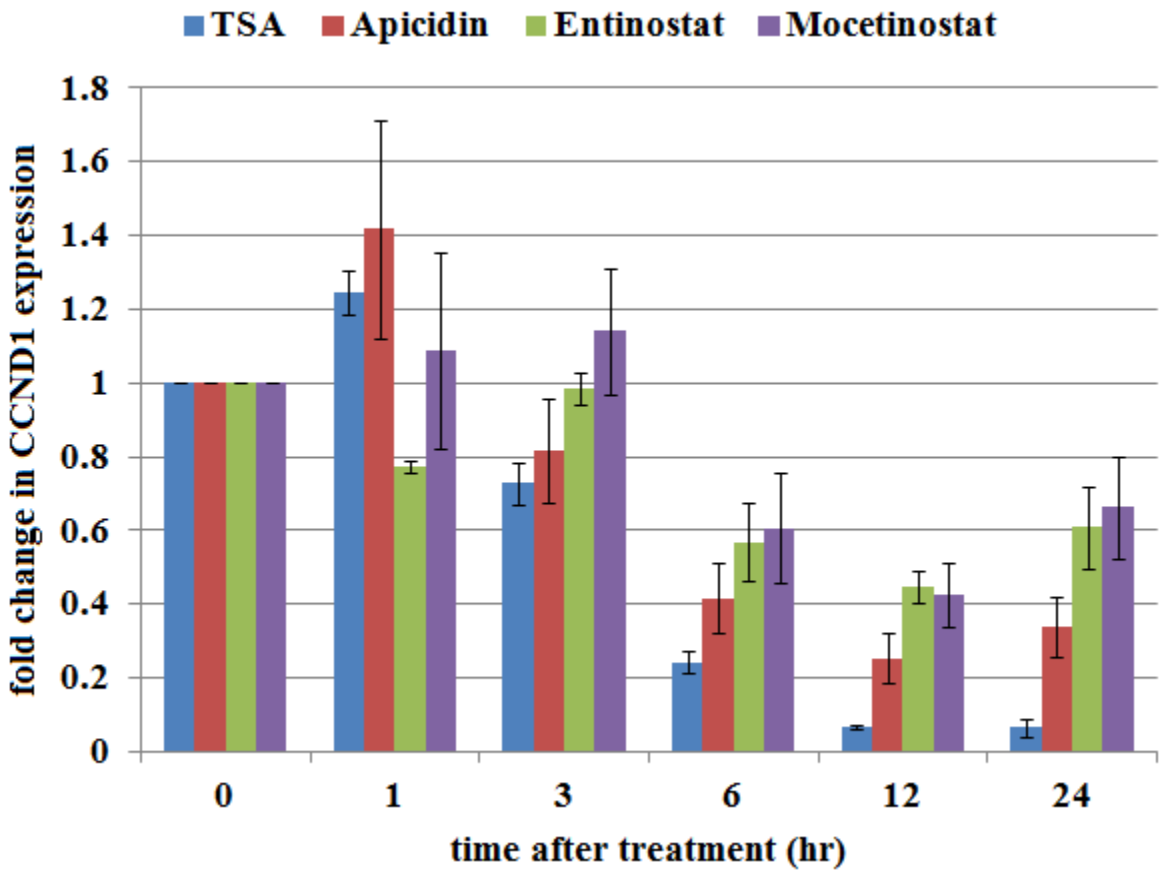

Figure 4.21. CCND1 repression in HDI-treated T47D breast cancer cells. Total RNA from untreated and 1 μM TSA, 2 μM Apicidin, 2 μM Entinostat or 1 μM Mocetinostat treated T47D cells were collected at the indicated time points. Samples were subjected to Real Time PCR with gene-specific primers, and the fold changes calculated by the ΔΔCt method. Data shown is the averaged results of technical repeat(s) +/- SEM.

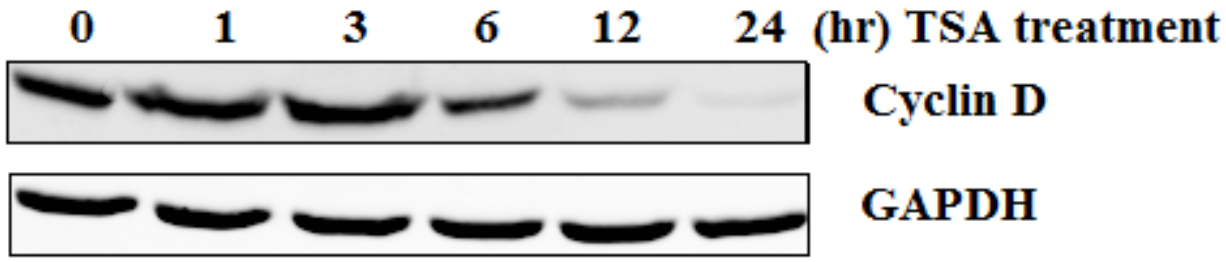

Figure 4.22. Cyclin D expression in TSA-treated T47D cells. The cells were treated with 1 µM TSA over a period of 24 hr and collected in 2X Laemmli sample buffer at the indicated time points. Following SDS-PAGE electrophoresis, 25 µg protein was transferred to a nitrocellulose membrane and incubated with anti-cyclin D antibody. GAPDH was assessed as a loading control. The data shown is representative of two biological repeats, which gave similar results.

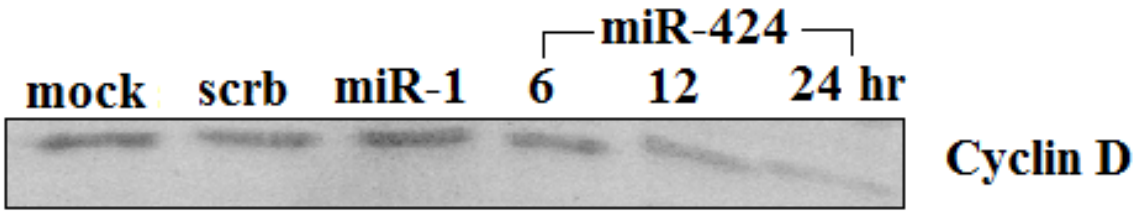

Figure 4.23. miR-424 transfection repressed cyclin D levels in T47D cells. The cells were seeded at 2 x 10^4 cells in 12-well plates and incubated with 300 nM synthetic miR-424 and Lipofectamine 2000 for 4 hr. RPMI-1640 media containing 20% FBS was added to the cells, and they were harvested over 24 hr in 2X Laemmli sample buffer. 25 µg of protein was resolved on a SDS-PAGE gel and immuno-blotting against cyclin D was performed. A mock control containing only Lipofectamine, a scrambled control and a non-specific miR-1 control were also included.

reduced in T47D and Hs578T cells (Figure 4.24), whereas expression was induced in the HCC 1419 cell line (data not shown). It is interesting to note that Apicidin, a class I-specific HDI thought not to target class II HDACs, resulted in modest repression of HDAC5 protein.

To eliminate HDI-mediated repression of HDAC5 protein levels, miR-9-3p was transfected into the T47D and Hs578T cell lines. The protein expression of HDAC5 in the Hs578T cell line was induced within 24 hr of miR-9-3p transfection, while data from the T47D cells demonstrated that miR-9-3p transfection had no effect on expression (data not shown). Therefore, it is likely that the observed HDAC5 repression observed within the HDI-treated cells is due to mechanisms independent of miR-9-3p induction in these particular cell lines.

4.3.3 Summary

The miRNA data further highlights the differential cellular effects HDIs have upon gene expression in the four representative breast cancer cell lines. The expression of miR-129-5p, miR-424 and/or miR9-3p expression increased in the T47D, Hs578T and HCC-1419 cell lines. Therefore, our results indicate that certain HDIs lead to induction of these tumour suppressive miRNA genes in a cell-specific manner. This induction of miRNA correlated with decreased mRNA stability and/or translational inhibition of certain targets. miRNA transfections validated Chek1 and cyclin D as putative miR-424 targets in the T47D cell line.

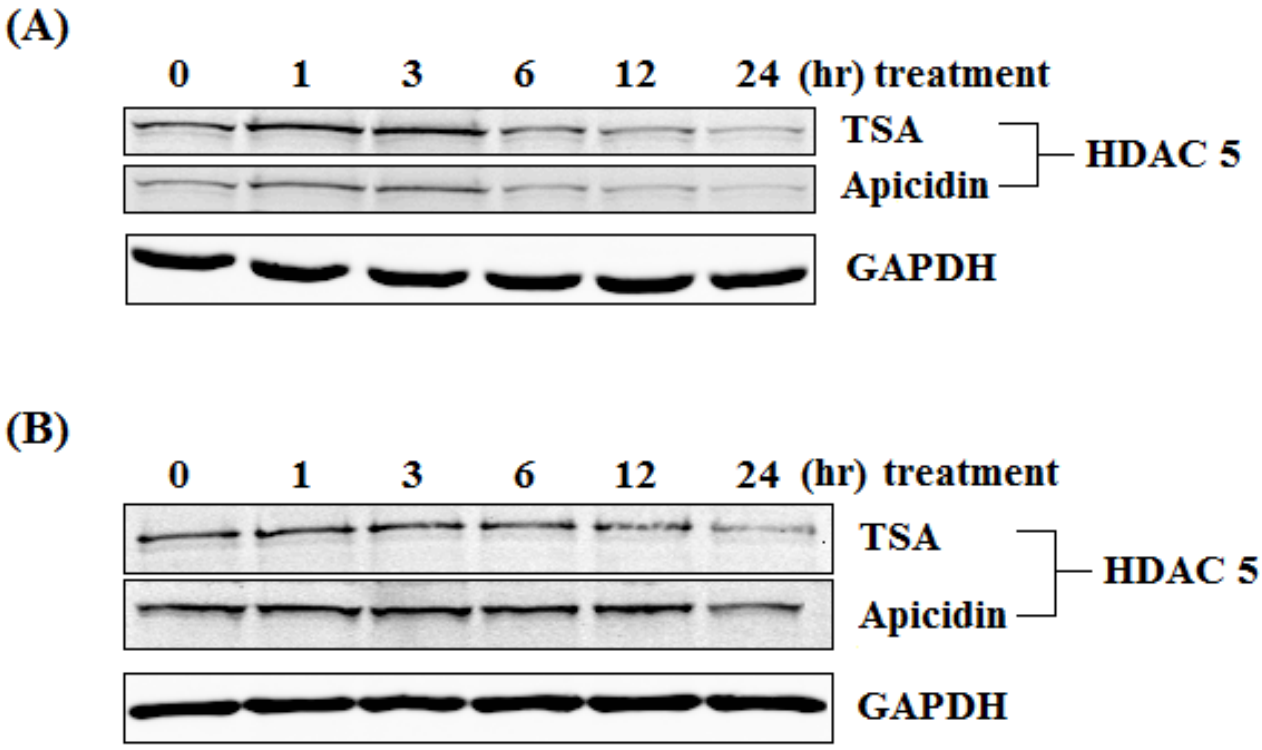

Figure 4.24. HDAC5 expression in HDI-treated T47D or Hs578T cells. (A) T47D or (B) Hs578T cells were treated with 1 µM TSA or 2 µM Apicidin over a period of 24 hr. Cells were harvested in 2X Laemmli sample buffer and 25 µg protein was resolved via SDS-PAGE. Immuno-blotting against Chek1 and GAPDH as a loading control was performed. The data shown is representative of two biological repeats, which gave similar results.

5. DISCUSSION

Histone deacetylase inhibitors are a novel class of anti-neoplastic drugs that mediate apoptosis, inhibit cell cycle progression, induce mitotic abnormalities and up-regulate gene expression in a wide variety of cancer-derived cell lines. While they were traditionally thought to act by inhibiting HDAC enzymes and increasing histone acetylation, the additional mechanisms by which HDIs exert their cellular effects are poorly understood. The inhibition of HDACs are widely associated with gene activation, however HDI treatment activates only a small percentage of human genes (2-10%), and an equal percentage are also repressed upon treatment (Van Lint *et al*., 1996; Gray *et al*., 2004; Marks *et al*., 2004; Mitsiades *et al*., 2004; Peart *et al*., 2005; Ropero and Esteller, 2007; LeBonte *et al*., 2009).

The FDA has approved two chemotherapeutic HDIs, Vorinostat in 2006 and Romidepsin in 2009, for the treatment of cutaneous T-cell lymphoma (Mann *et al*., 2007; Slingerland *et al*., 2013). Furthermore, following a recent phase II clinical study, the FDA has designated Entinostat as a 'breakthrough therapy' in ERα-positive metastatic breast cancers when combined with the aromatase inhibitor exemestane (Yardley *et al*., 2013). Numerous additional HDIs have exhibited promising clinical responses in animal studies and phase I/II clinical trials (Piekarz *et al*., 2007; Lee *et al*., 2008; Jagannath *et al*., 2010; Munster *et al*., 2011; Yardley *et al*., 2013).

Research completed in the Bonham Lab has demonstrated that TSA- and NaB-dependent repression of the proto-oncogene SRC is mediated through both the 1A and 1α promoters. This phenomenon has been observed in many cancer-derived cell lines, as well as in response to Apicidin treatment. While TSA is considered a 'pan-specific' HDI and inhibits both class I and class II enzymes, Apicidin is specific for class I (HDACs 1-3, 8) (Fournel *et al*., 2002; Furumai *et al*., 2002; Haggarty *et al*., 2003; Khan *et al*., 2008). ChIP analysis of the SRC promoter following TSA treatment indicated increased RNA pol II presence with increased H3 acetylation and H3K4 tri-methylation. In contrast, H3K36 tri-methylation, a marker of elongation, decreased across the coding region of the gene while occupancy of NELF increased at the promoter. It is thought that this HDI-dependent repression is mediated through promoter proximal pausing, although the exact mechanism behind this has yet to be determined. Interestingly, two class I-specific benzamides, Entinostat and Mocetinostat, were unable to down-regulate SRC expression, despite these compounds inducing the cyclin-dependent kinase inhibitor $p21^{WAF1}$. This potentially indicates that HDI-mediated repression of gene expression is not due to histone

acetylation, but rather non-transcriptional effects of the inhibitors, and highlights the potential complex nature of this class of drugs.

Therefore, to determine whether the repression of SRC and MYC is a cell-specific or a ubiquitous event, the responses of these proto-oncogenes to various classes of HDIs were surveyed in a panel of breast cancer cell lines. These inhibitors included the 'pan-inhibitory' TSA (hydroxamate) and three class I-specific drugs, Apicidin (cyclic tetrapeptide), Entinostat (benzamide) and Mocetinostat (benzamide). In addition, breast cancer cell lines were chosen based on their diverse molecular subtypes; this included T47D (ERα- and PgR-positive), Hs578T (triple-negative), BT-474 (ERα- and PgR-positive, and HER2-amplified) and HCC-1419 (HER2-amplified) (Neve *et al*., 2006; Grigoriadis *et al*., 2012).

5.1 The Response of Breast Cancer Cell Lines to Histone Deacetylase Inhibitors

5.1.1 All Classes of Histone Deacetylase Inhibitors Exhibit Cytotoxicity, and Induce Histone Acetylation and $p21^{WAF1}$ Expression

It has been demonstrated widely in the literature that HDIs induce cytotoxicity, histone acetylation and $p21^{WAF1}$ expression in cancer-derived cell lines (Vigushin *et al*., 2001; Glaser *et al*., 2003; Beckers *et al*., 2007; Ueda *et al*., 2007; Im *et al*., 2008; Fournel *et al*., 2008; Khan *et al*., 2008; Ahn *et al*., 2009; Kelly and Cowley, 2013). The cellular effects of these class-specific drugs shared several properties in the four breast cancer cell lines, inducing histone H3 acetylation and expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$, while decreasing cell viability. However, some of these compounds had moderate effects on these three responses.

5.1.1.1 Cytotoxicity

The cell viability profiles illustrate that HDIs exert their cytotoxic effects 48 hr following treatment. The efficacy of Entinostat has been demonstrated in phase II clinical studies and it has been considered 'breakthrough therapy' in ERα-positive metastatic breast cancer (Yardley *et al*., 2013). Reflecting the value of this compound in ERα-positive breast cancers, it was demonstrated that the cytotoxic properties of Entinostat in ERα-negative and/or HER2-amplified

cell lines were not as effective. In addition, while Apicidin is also a class I-specific HDI, its cytotoxic profile more closely resembled that of TSA in the majority of the cell lines. Therefore, even similar class I-specific HDIs such as Apicidin, Entinostat and Mocetinostat exhibit differential cytotoxic properties between cell lines.

Furthermore, the HER2-enriched HCC-1419 cell line was less sensitive to the cytotoxic effects mediated by these four compounds. Cellular signalling cascades mediated through the HER2/*neu* receptor could be responsible for this divergence; thus, treatment of non-HER2-enriched breast cancers with these chemotherapeutics could harbour greater clinical efficacy compared to treatment in HER2-amplified cancers. In addition, it has been reported in the literature that ERα-negative cell lines are less susceptible than ERα-positive cell lines to TSA-mediated growth inhibition (Margueron *et al*., 2004). Similar results were found in this study, with the Hs578T cell line exhibiting less sensitivity toward the cytotoxic effects of TSA.

These data indicate that the cell lines differentially respond to the various inhibitors, and that certain drugs possess higher potency to induce cancer-cell death. It has previously been established in the literature that these inhibitors can activate genes associated with different cellular functions (Halsall *et al*., 2012; Chatterjee *et al*., 2013). It is likely that, through HDAC inhibition or the acetylation of pro-apoptotic and cell cycle regulators, these compounds can influence the stability and downstream effects of a diverse set of proteins. This would ultimately lead to diverse responses in the cell following HDI treatment. In addition, the pharmacokinetics of these individual compounds and the presence or absence of efflux drug pumps on cellular surfaces may further influence the cellular response to these drugs.

5.1.1.2 Histone Acetylation

The induction of histone H3 acetylation following treatment illustrated that these compounds increased acetylation in a time- and dose-dependent manner in the four cell lines. While the relative HDI-mediated acetylation cannot be directly compared between the cell lines, several divergent responses can be highlighted. The transient properties of TSA in the Hs578T and HCC-1419 cell lines potentially indicate the rapid metabolism of the hydroxamate, whereas class I-specific inhibitors could have a longer half-life within the cells. The prolonged effects of

Apicidin, Entinostat and Mocetinostat may potentially lead to secondary responses in gene expression and/or cellular signalling.

While the benzamides inhibit the same complement of HDAC enzymes and had similar cytotoxic profiles in the Hs578T cell line, 0.5 µM Mocetinostat increased the acetylation of histones, whereas a 5 µM dose of Entinostat was required for enhanced histone acetylation. This potentially implicates Mocetinostat as an advantageous chemotherapeutic agent compared to Entinostat for in ERα-negative cancers, although additional studies would be required to validate this observation. In addition, Mocetinostat treatment induced cytotoxic effects at a lower concentration than Entinostat in this particular cell line, further suggesting the advantageous nature of this chemotherapeutic.

5.1.1.3 $p21^{WAF1}$ Induction

It has been demonstrated extensively in the literature that $p21^{WAF1}$ is induced in many-cancer-derived cell lines upon HDI treatment (Sowa *et al*., 1997; Huang *et al*., 2000; Lagger *et al*., 2003; Ocker and Scheider-Stock, 2007; Gartel and Tyner, 2002; Simboeck *et al*., 2010). The expression of $p21^{WAF1}$ was therefore examined in these cell lines following drug treatment. While the drugs globally induced $p21^{WAF1}$ over 24 hr, it is interesting to note the BT-474 cells responded with an immediate rapid, albeit transient, induction of expression unique to this cell line. While $p21^{WAF1}$ expression returned to control levels after 6 hr or 12 hr treatment, Entinostat exhibited a biphasic response. This may be due to secondary effects within the cell mediated by Entinostat that result in the up-regulation of $p21^{WAF1}$ expression, such as the activation of downstream signalling pathways that could stabilise mRNA expression or induce transcription. In contrast, $p21^{WAF1}$ expression in the T47D, Hs578T and HCC-1419 cell lines exhibited sustained induction over 12 hr or 24 hr treatment. $p21^{WAF1}$ has been implicated in the anti-proliferative effects of HDIs (Sherr and Roberts, 1999; Gartel and Tyner, 2002), and therefore anti-neoplastic agents drugs that exhibit prolonged $p21^{WAF1}$ induction could be therapeutically beneficial compared to those that have short-term effects.

The data also indicates that certain classes of HDIs are poor inducers of $p21^{WAF1}$ expression in certain breast cancer cell lines. While the benzamides share a similar structure, Entinostat induced $p21^{WAF1}$ to a greater extent than Mocetinostat in the T47D, Hs578T and HCC-

1419 cell lines. The induction of $p21^{WAF1}$ is dependent on the phosphorylation of H3S10 prior to acetylation of H3K14 (Simboeck *et al*., 2010), and therefore it is probable that these drugs do not activate the $p21^{WAF1}$ promoter as efficiently as other compounds. In addition, the Ing2 subunit of the Sin3A co-repressor complex has been demonstrated to be disrupted by Vorinostat treatment (Smith *et al*., 2010; Sardiu *et al*., 2014). Vorinostat shares a highly similar structure to TSA, and it is possible that while TSA could dissociate Ing2 from Sin3A, the benzamides are unable to and thus do not effect $p21^{WAF1}$ expression in the same manner. Furthermore, it has been shown that inhibiting Ing2 induces $p21^{WAF1}$ (Larrieu *et al*., 2010).

In addition, treatment with the lower concentrations of 1 µM TSA, 2 µM Apicidin, 2 µM Entinostat or 1 µM Mocetinostat in the Hs578T cell line, which did not induce significant histone acetylation, exhibited moderate $p21^{WAF1}$ induction. Recent research has illustrated that global histone acetylation is a poor marker of HDI-mediated gene induction (Halsall *et al*., 2012). It has also been suggested that HDI treatment results in transient acetylation of genes poised in the transcriptionally active state (Rada-Iglesias *et al*., 2007; Wang *et al*., 2009). Therefore, the acetylation of histones could be a poor indicator of the cellular activity of HDIs within certain cell lines. The data also supports the induction of $p21^{WAF1}$ expression despite poorly enhanced histone acetylation following treatment. However, it remains to be determined if the minor up-regulation of $p21^{WAF1}$ expression, albeit statistically significant, is biologically significant. ERα-negative cell lines, such as the Hs578T cell line, have been demonstrated to exhibit differential sensitivity to HDAC inhibitors than ERα-positive cell lines (Margueron *et al*., 2004), and the data obtained from this study potentially indicates that this differential sensitivity may also be observed with gene induction. While the HDIs increased $p21^{WAF1}$ mRNA in a dose-dependent manner in this cell line, experiments using more concentrations would be required to confirm this trend.

5.1.2 Histone Deacetylase Inhibitors Differentially Impact SRC and MYC Expression

While widespread HDI-mediated enhanced histone acetylation and induction of $p21^{WAF1}$ expression has been determined in these cell lines, it has become increasingly demonstrated in the literature that these inhibitors affect numerous cellular processes independent of histone acetylation and transcriptional up-regulation (Vigushin *et al*., 2001; Butler *et al*., 2002; Glaser *et*

al., 2003; Noh *et al*., 2003; Bolden *et al*., 2006; Rosato *et al*., 2006; Ma *et al*., 2009; Chatterjee *et al*., 2013). Although it had generally been accepted that HDI-mediated induction of transcription resulted from increased histone acetylation at gene promoters (Tsai *et al*., 2001), recent reports have revealed that many HDI-induced genes are in fact protected from enhanced acetylation (Halsall *et al*., 2012). Therefore, it has become increasingly evident that the effects of these compounds are not as straight-forward as initially thought. In addition, the various mechanisms of HDI-mediated transcriptional repression have yet to be fully elucidated.

In order to examine HDI-mediated effects on gene repression, SRC and MYC transcript levels were analysed following drug treatment in the four breast cancer cell lines. These two proto-oncogenes are frequently over-expressed in cancer (Dubik *et al*., 1987; Bonilla *et al*., 1988; Garcia *et al*., 1989; Cartwright *et al*., 1989, 1990; Dehm and Bonham, 2004; Wheeler *et al*., 2009; Horiuchi *et al*., 2012), and it is thought that certain classes of these chemotherapeutics could have a clinical advantage compared to other compounds due to their ability to repress SRC and MYC. It has previously been demonstrated in the Bonham Lab that SRC is repressed upon HDI treatment in many cancer-derived cell lines (Dehm *et al*., 2001); however, the extent of this HDI-mediated repression is unclear. In addition, these inhibitors cannot universally repress SRC and MYC levels, and treatment with the benzamides had no effect on expression (Bonham and Beaton-Brown, unpublished data). While SRC and MYC share similar promoter elements (Henricksson and Luscher, 1996; Facchini and Penn, 1998; Xiao *et al*., 1998; Bonham *et al*., 2003, 2000; Levens, 2008), the mechanisms of HDI-mediated transcriptional repression between these two proto-oncogenes are unique. The data presented in this thesis suggests that SRC and MYC are not globally repressed following HDI treatment in these breast cancer-derived cell lines.

5.1.2.1 The Amplification and/or Rearrangement of the MYC Locus and HDI-Mediated Repression

While MYC expression is repressed following HDI treatment in many cancer-derived cell lines (Wang *et al*., 1998; Sasakawa *et al*., 2003; Xu *et al*. 2005; Ierano *et al*., 2013, Leone *et al*., 2015; Raha *et al*., 2015), this down-regulation was not observed following treatment in the Hs578T and HCC-1419 cell lines. The MYC gene is amplified in many triple-negative breast

cancer cell lines, and the Hs578T and HCC-1419 cell lines both possess an amplified MYC genomic region as well as numerous other genomic alterations (Kao *et al*., 2009); this amplification is also reflected in the elevated steady-state levels of MYC in these cell lines. The rearrangement and/or amplification of the MYC locus could alter the normal response of the promoter to these inhibitors, and is likely responsible for the abnormal expression of MYC following treatment in these two cell lines. The T47D and BT-474 cell lines do not harbour MYC amplification and/or rearrangement at the genomic level, although the steady-state expression of MYC is comparable to the HCC-1419 cell line. This increased expression could be due to enhanced transcription without genomic amplification.

While it is more likely that the rearrangement of the MYC locus is responsible for the abnormal response to the HDIs, it is also possible that the inhibition of class II HDACs and/or the cellular signalling pathways activated or inactivated by these inhibitors could differentially influence transcription. Furthermore, the possible detrimental effects of MYC induction in breast cancer patients with a rearranged and/or amplified MYC locus remain unclear, and therefore further studies with MYC-amplified cell lines are required. In fact, it is possible that in certain oncogenic backgrounds with MYC over-expression, the induction of $p21^{WAF1}$ could lead to cell cycle progression rather than cell cycle arrest through the enhancement of cyclin D (Bearss *et al*., 2002). Additionally, experiments involving more cell lines with abnormal MYC loci would verify the altered response of MYC in cell lines or tumours with genomic rearrangements.

5.1.2.2 The Effects of HDAC Inhibition on SRC and MYC Repression

The inhibitors utilised in this study target different classes of HDAC enzymes; Apicidin and the two benzamides, Entinostat and Mocetinostat, inhibit the class I HDACs with varying specificities, while TSA is 'pan-inhibitory' and targets class I, II and IV HDACs (Fournel *et al*., 2002; Furumai *et al*., 2002; Haggarty *et al*., 2003; Khan *et al*., 2008). It is possible that these distinct properties contribute to the HDI-specific effects on SRC and MYC expression between the cell lines. For example, while the benzamides and Apicidin are class I-specific HDAC inhibitors and can catalytically inhibit HDAC1, HDAC2 and HDAC3, they differentially impacted SRC and/or MYC activation and/or repression. However, these differences were not consistent between the various cell lines, and determining the specific HDAC responsible is

challenging.

In addition, it has been demonstrated that benzamides preferentially inhibit the HDAC3-containing complex NCoR/SMRT, while the HDAC1/2-containing Sin3 complex is targeted by the hydroxamates and cyclic tetrapeptides. While these inhibitors can inhibit the catalytic activity of the HDAC subunit, it is becoming increasingly apparent that HDIs interact differentially with these multi-protein co-repressors complexes (Bantscheff *et al*., 2011). These differences in selective targeting and enhanced binding to certain co-repressors may also account for the differential responses observed following HDI treatment. Furthermore, the cell lines could express a unique complement of HDAC complexes and/or protein subunits, modifying the impact that the same HDI could have within different cell lines.

It is also probable that the differential effects of these compounds are due to the acetylation and destabilisation of transcription factors and/or protein complexes associated with HDAC co-repressors, rather than direct HDAC inhibition. TSA and Apicidin could induce acetylation of transcription factors, whereas Entinostat and Mocetinostat have no effect, resulting in varied downstream responses. Furthermore, TSA has been shown to disrupt HDAC and protein phosphatase complexes (Chen *et al*., 2008), and this could alter the chromatin-reading proteins at the promoters of genes, thereby altering gene expression following HDI treatment in these cells. In addition, down-regulating the Sin3A co-repressor complex leads to the repression of MYC-target genes (McDonel *et al*., 2011); it is likely that the compounds which disrupt this complex (Sardiu *et al*., 2014) contribute to this process. While the specific HDAC(s) or complex(es) responsible for repression and/or activation of these proto-oncogenes following HDI treatment cannot be determined, it is apparent that the actions of these inhibitors are widespread and unpredictable.

5.1.2.3 The Pharmacokinetics of HDIs in the Breast Cancer Cell Lines

The pharmacokinetics of these drugs within the cells could impact their effects on gene expression. The transient nature of HDI-mediated SRC repression in certain cell lines could be due to the rapid metabolism and/or clearance from the cells. For instance, the BT-474 cell line exhibited transient histone H3 acetylation and SRC repression following TSA treatment, and therefore this inhibitor may have a short half-life within this cell line. A rapid clearance of TSA

from the cell could account for its transient nature. It is also possible that these cells uptake the drugs with various affinities, and this could impact the HDI-mediated effects observed in gene expression, as well as cytotoxicity and histone acetylation.

5.1.2.4 HDI-Mediated Promoter Proximal Pausing

Work from the Bonham Lab has indicated that TSA-mediated repression of SRC and MYC in the T47D cell line is likely due to promoter proximal pausing of RNA pol II (Bonham and Beaton-Brown, unpublished data). The chromatin markers associated with the gene promoters following drug treatment supports this hypothesis. While the data presented in this thesis further support TSA-mediated SRC repression in the Hs578T, BT-474 and HCC-1419 breast cancer cell lines, MYC is not subjected to the same regulatory mechanisms in these cell lines. While this could be due to the rearrangement and/or amplification of the MYC locus in the Hs578T and HCC-1419 cell lines, as previously mentioned, it is also probable that these drugs possess different cellular targets in the diverse cell lines. The data suggest the repression of these proto-oncogenes are potentially due to multiple mechanisms, and are cell-specific and not a universal phenomenon.

5.1.2.5 The Clinical Relevance of SRC and MYC Repression in Molecular Subtypes of Breast Cancer

These cell lines represent the different molecular subtypes of breast cancer (Neve *et al*., 2006; Grigoriadas *et al*., 2012), and it has been demonstrated that cell lines recapitulate the heterogeneous nature of breast cancer (Neve *et al*., 2006). In addition, the down-regulation of SRC expression has been associated with reduced tumorigenic potential (Ishizawar *et al*., 2004; Hiscox *et al*., 2006; Park *et al*., 2008; Vandyke *et al*., 2009; Montero *et al*., 2011) in cell lines.

The SRC expression data obtained in this study suggest that prolonged exposure to the benzamides in the HER2/*neu*-amplified HCC-1419 cell line could mediate induction of SRC, activating Src-dependent signalling pathways downstream. This could indicate cell-specific control of SRC transcription dependent upon specific signalling cascades that result from enhanced HER2/*neu* activity. However, the BT-474 cell line also over-expresses HER2/*neu*, but their response to the compounds are divergent from that observed in the HCC-1419 cells. This

could be due to additional HDI-mediated, non-transcriptional effects on cellular signalling, which are present in the ERα-positive BT-474 cells but absent from the ERα-negative HCC-1419 cells. Therefore, additional experiments with HER2-amplified cell lines as well as *in vivo* studies would need to be performed to identify any clinical disadvantages of Entinostat and Mocetinostat in this particular subtype.

In addition, the BT-474 cell line exhibited the lowest steady-state levels of SRC and MYC expression. Therefore, the down-regulation of these proto-oncogenes may not be biologically significant in this cell line, or clinically significant in primary tumours with lower SRC and MYC expression. In contrast, the T47D cell line expressed high levels of SRC mRNA, and repression observed following TSA and Apicidin treatment, whereas Entinostat and Mocetinostat, two benzamides currently used in ERα-positive tumour treatment and in phase I/II clinical trials, had no effect on expression. This suggests that certain classes of these chemotherapeutics are advantageous compared to others due to their ability to repress these genes in certain subtypes of breast cancer.

Furthermore, while the T47D and BT-474 cell lines are both ERα-positive, they exhibited differential repression of SRC following Mocetinostat and Entinostat treatment. In contrast, the expression of MYC following treatment with these benzamides showed similar results between the two cell lines. The presence or absence of ERα had no apparent influence on the HDI-mediated repression of these proto-oncogenes.

The data presented in this thesis indicate that different molecular subtypes of breast cancer could clinically benefit from treatment with certain chemotherapeutics. While TSA repressed SRC in all four cell lines, MYC repression was only observed in the ERα-positive cell lines, potentially indicating that 'pan-specific' inhibitors related to this compound have an advantage over benzamide-derived compounds. Entinostat and Mocetinostat induced or had no effect on MYC expression, which could be clinically disadvantageous. However, it should be clarified that although these trends were observed within the ERα-positive, triple-negative or HER2/*neu* amplified cell lines, it cannot be determined if these are universal effects common to all breast cancer cell lines of these subtypes. Investigations in additional cell lines would be required to draw definitive conclusions to these particular subtypes.

5.2 Histone Deacetylase Inhibitors Mediate miRNA Expression

miRNA genes are located within genomic regions associated with cancer and can act in a tumour suppressive or oncogenic manner (Lu *et al*., 2005; Ma *et al*., 2007; Babashah and Soleimani, 2011; Tsai *et al*., 2011). Furthermore, HDI treatment has repressed or induced miRNA gene expression in many cancer-derived cell lines (Scott *et al.*, 2006; Lu *et al.*, 2005; Tsai *et al*., 2011). It was hypothesised that these compounds differentially affected miRNA expression in the breast cancer cell lines. While miRNA expression was up-regulated with HDI treatment, these observations were not recapitulated from each cell line.

5.2.1 miR-424 and miR-129-5p Induction

Both miR-424 and miR-129-5p are anti-proliferative tumour suppressor miRNA, and are frequently down-regulated in cancers (Wu *et al*., 2010; Xu *et al*., 2012). In fact, miR-129-5p is repressed in many malignancies (Dyrskjøt *et al*., 2009; Wu *et al*., 2010), and induction has been demonstrated with TSA and Vorinostat treatment (Bandres *et al*., 2009; Brest *et al.*, 2011). The data presented in this thesis demonstrate similar results, wherein several of these inhibitors induced miR-129-5p expression, particularly TSA and Entinostat treatment in the T47D cells. Furthermore, recent research in thyroid cancer cells indicates that miR-129-5p is necessary for HDI-mediated cell death (Brest *et al*., 2011). Therefore, HDI-mediated induction of miR-129-5p in the T47D cell line could be therapeutically beneficial and lead to apoptosis, suggesting an advantage over those inhibitors which do not induce miR-129-5p.

In addition, miR-424 is also a tumour suppressor (Lui *et al*., 2008; Pallasch *et al*., 2009; Xu *et al*., 2012), and its induction could likewise be clinically beneficial. In fact, it has been illustrated in the literature that miR-424 up-regulation in cervical cancer cell lines leads to apoptosis and cell cycle arrest. The inhibition of the cell cycle at the G1/S phase is likely mediated by the down-regulation of the protein checkpoint kinase Chek1. (Xu *et al*., 2012). Similar results were obtained in the T47D cell line, wherein miR-424 transfection decreased Chek1 protein levels. This suggests a putative role for miR-424 as a tumour suppressor miRNA in certain breast cancers. Therefore, the ability of Entinostat and TSA to significantly induce miR-424 in the T47D cell line could contribute to the anti-tumorigenic effects these HDIs exhibit

in cancerous cells.

The induction of these tumour suppressors could be clinically advantageous in certain breast cancers, and chemotherapeutics which induce miR-129-5p and/or miR-424 may be better candidates in cancer treatment. Entinostat has shown promising results in ERα-positive breast cancers, and treatment induced miR-424 and miR-129-5p expression in an ERα-positive cell line. Interestingly, miRNA expression in two HER2/*neu* amplified cell lines (BT-474 and HCC-1419) were not induced with the inhibitors, and therefore miR-424 and/or miR-129-5p induction could be an important therapeutic avenue in non-HER2/*neu* amplified breast cancers. It is also possible that HER2/*neu* signalling has a role in the transcription and/or biogenesis of these miRNA, and contributes to their repression. Furthermore, while Apicidin, Entinostat and Mocetinostat are class I-specific drugs and inhibit the same HDAC enzymes, they did not have similar effects on miRNA expression. This discrepancy could be due to the inhibitors targeting different co-repressor subunits or through altered miRNA biogenesis.

While HDI-mediated up-regulation of miR-424 and miR-129-5p correlated with repression of their gene targets (cyclin D, Chek1, ERα and c-MYB) in the T47D and/or Hs578T cell lines, the expression of other validated targets were not down-regulated. The time course experiments were terminated at 24 hr due to the cytotoxicity of the HDIs and resultant poor quality of the samples at 48h. It is possible that this 24 hr time period is too brief to observe miRNA-mediated protein down-regulation. However, analysis of cells transfected with miRNA was extended to 72h to allow the biogenesis of the miRNA molecules and downstream effects. The cyclin D and Chek1 proteins were down-regulated following transfection and therefore confirmed as miR-424 targets (Wu *et al.*, 2010; Xu *et al.*, 2012) in the T47D cell line.

It has been demonstrated in the literature that cyclin D expression is down-regulated following both HDI treatment (Alao *et al.*, 2006; Zhang *et al.*, 2012; Jin *et al.*, 2012) and miR-424 induction (Wu *et al.*, 2010). It is therefore probable that multiple regulatory mechanisms exist to repress cyclin D, and miR-424 only plays a role in this process. Furthermore, it could be concluded that down-regulation of c-MYB and ERα at the mRNA and/or protein level were due to either direct HDI-mediated effects, or a combination of direct and miRNA-mediated effects. Nonetheless, the up-regulation of these tumour suppressor miRNA by certain HDIs resulted in the down-regulation of their target genes, thus contributing to the anti-cancerous effects of these drugs.

5.2.2 miR-9-3p Induction

While little is known on the role of miR-9-3p in cancer progression, it was found to be down-regulated in B-cell lymphoma and breast cancer cell lines (Roccaro et al., 2010; Zawistowski *et al*., 2013). Furthermore, it is interesting to note that miR-9-3p targets the 3' UTR of HDAC4 and HDAC5 (Roccaro *et al*., 2010), both class II(a) HDACs which can be targeted by 'pan-specific' HDIs. Due to this, it could be postulated that treatment with the 'pan-specific' TSA would have the highest impact on miR-9-3p expression, and potentially be one mechanism by which these drugs down-regulate HDAC class II(a) activity. Due to the proposed role of miR-9-3p in acetylation and deacetylation processes (Roccaro *et al.*, 2010), it could be suggested that the induction of miR-9-3p is due to class IIa involvement. This is supported by the 'pan-specific' TSA substantially inducing expression, whereas the class I-specific inhibitors were more moderate in their effects.

However, miR-9-3p transfection in the T47D and Hs578T cell lines had either no effect or up-regulated HDAC5 protein expression, respectively. Due to the promiscuous nature of miRNA, it has been suggested that these small non-coding molecules exert cell-specific regulatory mechanisms on mRNA translation and/or decay (Bartel *et al*., 2004; Bagga *et al*., 2005). It is possible in these breast cancer cell lines that miR-9-3p preferentially targets the 3' UTR of HDAC4 or has different gene targets. In addition, it is likely that HDAC5 protein repression observed with TSA treatment is due to regulatory mechanisms that are independent to miR-9-3p induction, such as the effect of the HDIs on transcription or cell signalling.

6. CONCLUSIONS AND FUTURE STUDIES

The heterogeneity of breast carcinoma has been demonstrated in studies examining the clinical and molecular characterisation of the disease. These malignancies arise through myriad abnormal cellular processes, including imbalances in histone acetylation by the reciprocally acting HAT and HDAC enzymes. The dysregulation of these epigenetic mechanisms represents a novel level of gene control, which can be exploited by various chromatin-remodelling chemotherapeutics. Histone deacetylase inhibitors are a novel class of these chemotherapeutics that preferentially affect cancerous cells, leading to apoptosis, cell cycle arrest and/or differentiation (Mehnert *et al*., 2007; Ma *et al*., 2009; Wanczyk *et al*., 2011). While these drugs were traditionally thought to shift the balance of HAT and HDAC activity, thereby altering chromatin modifications and enhancing gene expression, recent evidence suggests that the actions of HDIs are more complex than originally thought. They have been shown to exhibit cell-specific and/or differential effects in a variety of cancer-derived cell lines (Gray *et al*., 2004; Ropero and Esteller, 2007; LeBonte *et al*., 2009; Halsall *et al*., 2012; Chatterjee *et al*., 2013).

The data presented in this thesis further highlights the complex nature and unpredictability of these inhibitors. It also highlights the clinical advantages that certain drugs could have over other chemical classes of these compounds for the treatment of certain breast tumours. It has previously been demonstrated that the SRC proto-oncogene is repressed following TSA, NaB and/or Apicidin treatment in many cancerous cell lines, including colorectal, breast and hepatocellular (Kostyniuk *et al*., 2002; Dehm and Bonham, 2004; Hirsch *et al*., 2006; Bonham and Beaton-Brown, unpublished data). The repression of SRC is mediated from both the 1A and 1α promoters despite increased histone acetylation at these regions, and does not require *de novo* protein synthesis. This HDI-mediated repression was likewise observed for the MYC proto-oncogene. However, Entinostat and Mocetinostat treatment did not down-regulate SRC and MYC. These are two benzamides which are currently in clinical trials, and Entinostat has also been labelled a 'breakthrough therapy' in conjunction with exemestane in the treatment of metastatic ERα-positive breast cancers. It would be expected that chemotherapeutics that down-regulate SRC and/or MYC while inducing $p21^{WAF1}$ would be more potent drugs to treat breast tumours. In addition, the up-regulation of tumour suppressor miRNA is another avenue that can be exploited to induce cancer cell death and anti-neoplastic effects.

The data presented in this study indicates that HDAC inhibitors do not universally repress SRC or MYC in four cell lines representative of the heterogeneous nature of breast cancer. However, these compounds induced cell cytotoxicity, histone H3 acetylation and $p21^{WAF1}$ expression, indicating that the mechanisms of HDI-mediated repression are particular to certain compounds. While TSA and Apicidin repressed SRC in all cell lines, treatment with the benzamides had no effect. MYC repression was only observed following TSA treatment in the BT-474 and T47D cell lines, and the class I-specific inhibitors had either no effect or induced expression.

It has been demonstrated that benzamides are unable to inhibit HDAC activity within the Sin3 complex. In fact, while benzamides such as Entinostat can rapidly inhibit HDAC3 and HDAC3-containing co-repressor complexes, they have little effect on the Sin3 components and HDAC1/2 enzymes. However, they are able to inhibit the CoREST and NuRD complexes, which contain HDAC1 and/or HDAC2 dimers and HDAC3 (Bantscheff *et al*., 2011; Becher *et al*., 2014). The inhibition of these complexes can lead to increased histone acetylation and $p21^{WAF1}$ expression. In contrast, hydroxamates such as TSA and Vorinostat, rapidly inhibit the three co-repressor complexes Sin3, NuRD and CoREST (Becher *et al*., 2014), and therefore can also induce histone acetylation and $p21^{WAF1}$. The distinct kinetics of these inhibitors in affecting the HDAC co-repressor complexes could explain the differences in enhanced histone H3 acetylation, $p21^{WAF1}$ induction and cytotoxicity observed between the HDIs within the cell lines.

The divergence in the effects of these compounds on co-repressors and HDAC enzymes could also account for the differential effects observed between SRC and MYC repression following treatment with similarly structured HDIs. Certain drugs, such as TSA and/or Apicidin, could inhibit HDAC1/2 within the Sin3 complex and/or disrupt complex integrity. Therefore, it is possible that the Sin3 complex could be responsible for HDI-mediated repression of these proto-oncogenes. Indeed, Vorinostat dissociates Ing2 from the Sin3A complex, abrogating its activity (Smith *et al*., 2010). The mechanism(s) by which TSA and/or Apicidin exert these effects are currently unknown; it could be mediated through HDAC inhibition and/or complex disruption. In contrast, Mocetinostat and Entinostat cannot inhibit the HDACs within Sin3, and furthermore have no effect on the integrity of the complex (Figure 6.1).

In order to test whether the Sin3 complex is associated with the SRC and MYC promoters, ChIP experiments can be performed. If this co-repressor is involved in regulation, it

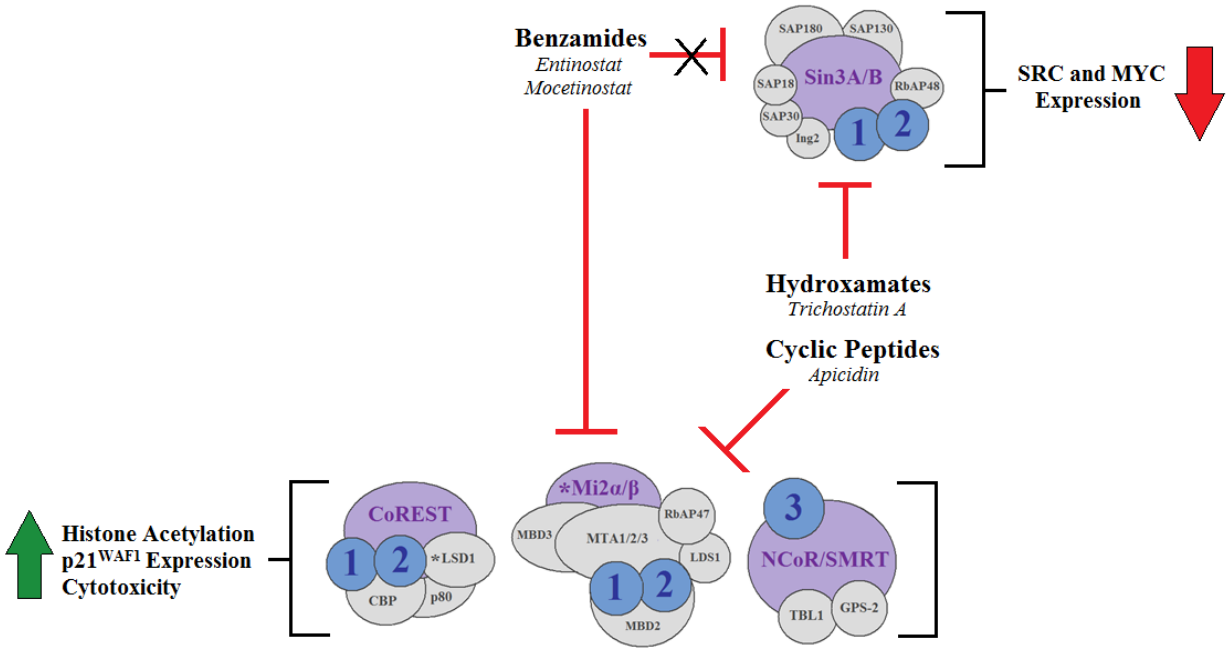

Figure 6.1. Model of HDI-mediated inhibition of HDAC co-repressor complexes. A red arrow indicates repression and a green arrow indicates activation. The benzamides have no effect on the Sin3 complex but can inhibit the CoREST, NuRD and NCOR/SMRT complexes. Hydroxamates and cyclic tetrapeptides can inhibit the four co-repressor complexes. Through these mechanisms, Entinostat, Mocetinostat, TSA and Apicidin activate gene expression and induce cytotoxicity and histone acetylation, whereas only TSA and Apicidin repress gene expression.

would be expected to be enriched at the promoter regions. The components of the Sin3 complex could be knocked down in cell lines, and the effects on SRC and MYC expression observed through RT-qPCR. These proto-oncogenes would be repressed following Sin3 disruption if the complex is involved in regulation. In addition, HDAC1 and/or HDAC2 can be over-expressed in the cell lines and the effect on SRC and MYC expression determined. While this experiment may implicate the HDAC1/2-containing Sin3 complex in gene regulation, it does not eliminate the consequences that HDAC over-expression could have in the cell. These may involve other cellular processes, which potentially could lead to differences in MYC and SRC expression. Despite the exact mechanism responsible for the HDI-mediated repression of SRC and/or MYC in these cell lines, it is evident that these classes of inhibitors are complex and unpredictable in nature.

In addition, it has been shown that RNA pol II-encoded miRNA are induced upon HDI treatment (Lu *et al.,* 2005; Scott *et al.,* 2006). miRNAs are implicated in numerous cellular processes important in malignant pathogenic processes and have been shown to have anti-tumour activity (Iorio *et al.,* 2005; Lu *et al.,* 2005; Scott *et al.,* 2006; Lowery *et al.,* 2009). The data presented in this thesis demonstrated that HDIs induced miR-129-5p, miR-424 and miR-9-3p expression in several breast cancer cell lines. These tumour suppressor miRNA were not up-regulated in all cell lines with all the inhibitors, and this further highlights the complexity and unpredictability that these compounds have within cell lines. It is likely that this up-regulation observed following TSA, Apicidin, Entinostat and/or Mocetinostat treatment is mediated through co-repressor complexes commonly affected by the four inhibitors. However, it is unclear whether the mechanisms of HDI-mediated induction are due to enhanced transcription from the miRNA promoters or altered miRNA biogenic processes. Therefore, analysing the expression of pri-miRNA and pre-miRNA through RT-qPCR techniques could eliminate HDI-mediated enhancement of miRNA processing. It is also probable that certain HDIs preferentially target certain miRNA for processing by Drosha and Dicer.

Furthermore, the cyclin D and Chek1 protein were down-regulated following miRNA transfection, suggesting that miR-424 targets the 3' UTR of these transcripts for translational inhibition and/or mRNA decay. To determine the mechanism by which this miRNA regulates cyclin D and Chek1 post-transcriptionally, luciferase reporter assays involving the 3' UTR of these transcripts and miRNA transfection can be utilised. The measurement of the luciferase

activity would indicate if the mRNA is degraded following transfection, or if the mechanism of regulation is through the inhibition of translation. miR-424 is involved in cell cycle arrest and apoptosis in cervical cancer (Xu *et al.*, 2012), and transfection experiments followed by TUNEL assays or Annexin V assays for apoptotic detection can be performed. This would therefore indicate the potential advantages of miR-424 induction in the breast cancer cell lines. In addition, transwell migration and invasion assays following miR-424 and miR-129-5p transfection to determine if the induction of these miRNA leads to a decrease in the metastatic properties of cells. It has also been demonstrated that miR-129-5p is required for HDI-mediated apoptosis in thyroid cancer cells (Brest *et al.*, 2011). Transfection of anti-miR-129-5p followed by HDI treatment and subsequent apoptotic assays could elucidate whether this miRNA is required for the HDI-mediated cytotoxicity observed in the breast cancer cell lines.

The field of research into the mechanisms of HDI action has evolved considerably, particularly in the last decade. Recent research has illustrated that these inhibitors influence more than merely histone acetylation and gene activation. The repression of gene expression and non-transcriptional effects are being demonstrated in many cancer-derived cell lines, and it has become apparent that HDIs are more multifaceted than previously thought. In fact, the complexity of these drugs and their unique interaction with HDAC enzymes and the co-repressor complexes could hinder the development of novel chemotherapeutics in the treatment of cancers. The data presented in this thesis further highlights the complex nature and unpredictability of these inhibitors. Furthermore, it provides a potential framework for the mechanisms by which the various HDAC inhibitors act through the co-repressor complexes and highlights the differential gene expression profiles resulting from these unique properties.

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